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(54) **Title:** THERAPEUTIC CALCIUM PHOSPHATE PARTICLES FOR USE IN INHIBITING EXPRESSION OF A GENE

(57) **Abstract:** Novel calcium phosphate core particles, methods of making them, and methods of using them as carriers of biologically active material and controlled release matrices for biologically active material are disclosed. The core particles may have a surface modifying agent and/or biologically active material, such as polynucleotide material partially coating the particle or impregnated therein or both. The particles may be used to carry polynucleotide (or oligonucleotide) material that is complementary to a sequence of mRNA that binds to or ligates with and inhibits the expression of particular genes (by blocking translation of the mRNA sequence). The core particles are substantially spherical in shape, and have a substantially smooth surface.

THERAPEUTIC CALCIUM PHOSPHATE PARTICLES FOR USE IN INHIBITING EXPRESSION OF A GENE

This application claims benefit of the filing dates of U.S. Provisional Application Serial No. 60/598,146 filed August 2, 2004, and it is a continuation of U.S. Serial No. 09/794,576 filed February 27, 2001 which claims priority to U.S. Patent Application Serial No. 09/496,771 filed on February 3, 2000 (now U.S. Patent No. 6,355,271), which claims benefit of the filing dates of U.S. Provisional Application Serial Nos. 60/118,356; 60/118,364; and 60/118,355, all filed February 3, 1999, the entire contents of each of which are hereby incorporated by reference.

BACKGROUND OF INVENTION

1. Field of the Invention

The present invention relates to novel calcium phosphate core particles, to methods of making them, and to methods of using them as carriers for biologically active material and as controlled release matrices for biologically active material. In particular, the calcium phosphate particles described herein may be used to deliver DNA or RNA vaccines, as well as deliver DNA or RNA sequences that inhibit or silence gene expression.

2. Description of Related Art

Nanometer scale particles have been proposed for use as carrier particles, as supports for biologically active molecules, such as proteins, and as decoy viruses. See U.S. Patent Nos. 5,178,882; 5,219,577; 5,306,508; 5,334,394; 5,460,830; 5,460,831; 5,462,750; and 5,464,634, the entire contents of each of which are hereby incorporated by reference.

The particles disclosed in the above-referenced patents, however, are generally small, in the 10-200 nm size range. Particles of this size are difficult to make with any degree of consistency, and their morphology is not described in any detail. None of these patents disclose the use of nanoparticles as sustained release matrices, as delivery mechanisms for vaccines, or as delivery mechanisms for nucleotide sequences that inhibit gene expression.

Therefore, an important need remains for calcium phosphate core particles that can be effectively used as supports and matrices for sustained release of polynucleotide material (DNA or RNA) encoding immunogenic polypeptides. There is also a need also for particles that can be used to deliver small inhibitory RNA, which is double stranded RNA that can be used to silence or inhibit gene expression.

With respect to DNA immunization, traditional vaccination involves exposing a potential host to attenuated or killed pathogens, or immunogenic components thereof (e.g.,

proteins or glycoproteins). The basic strategy has changed little since the development of the first smallpox vaccine nearly a century ago, although modern developments permit genetic engineering of recombinant protein vaccines. However, traditional vaccine methodologies may be undesirable as a result of their expense, instability, poor immunogenicity, limited heterogeneity and potential infectivity.

Polynucleotide vaccination presents a different vaccine methodology, whereby polynucleotide material, such as DNA or RNA, encoding an immunogenic polypeptide is delivered intracellularly to a potential host. The genetic material is taken up and expressed by these cells, leading to both a humoral and a cell-mediated immune response. It is not entirely clear whether DNA vaccines function as a result of integration or simply long-term episomal maintenance.

Polynucleotide vaccination provides numerous advantages over traditional vaccination. Polynucleotide vaccines eliminate the risk of infection associated with live attenuated viruses, yet advantageously induce both humoral and cell-mediated responses. Polynucleotide vaccines further provide prolonged immunogen expression, generating significant immunological memory and eliminating the need for multiple inoculations. Polynucleotide vaccines are very stable, permitting prolonged storage, transport and distribution under variable conditions. As a further advantage, a single polynucleotide vaccine may be engineered to provide multiple immunogenic polypeptides. Thus, a single DNA vaccine can be used to immunize against multiple pathogens, or multiple strains of the same pathogen. Finally, polynucleotide vaccines are much simpler and less expensive to manufacture than traditional vaccines.

Polynucleotide vaccines may take various forms. The genetic material can be provided, for example, in combination with adjuvants capable of stimulating the immune response. Administration of the DNA or RNA coated onto microscopic beads has been suggested. See J. J. Donnelly et al., *Annu. Rev. Immunol.* 15, 617 (1997). Various routes of administration are also possible, and may include, for example, intravenous, subcutaneous and intramuscular administration.

A desirable immune response to an immunogenic polypeptide is two-fold, involving both humoral and cellular-mediated immunity. The humoral component involves stimulation of B cells to product antibodies capable of recognizing extracellular pathogens, while the cell-mediated component involves T lymphocytes capable of recognizing intracellular pathogens. Cytotoxic T-lymphocytes (CTLs) play an important role in the latter, by lysing

virally-infected or bacterially-infected cells. Specifically, CTLs possess receptors capable of recognizing foreign peptides associated with MHC class I and/or class II molecules. These peptides can be derived from endogenously synthesized foreign proteins, regardless of the protein's location or function within the pathogen. Thus, CTLs can recognize epitopes derived from conserved internal viral proteins (J.W. Yewdell et al., *Proc. Natl. Acad. Sci.* (USA) 82, 1785 (1985); A.R. M. Towsend, et al., *Cell* 44, 959 (1986); A.J., McMichael et al., J. Gen. Virol. 67, 719 (1986); A.R. M. Towsend and H., *Annu. Rev. Immunol.* 7, 601 (1989)) and may therefore permit heterologous protection against viruses with multiple serotypes or high mutation rates. Polynucleotide vaccination can stimulate both forms of immune response, and thus is very desirable.

Efforts to use polynucleotide vaccination have focused on the use of viral vectors to deliver polynucleotides to host cells. J. R. Bennink et al., 311, 578 (1984); J. R. Bennink and J. W. Yewdell, *Curr. Top. Microbiol. Immunol.* 163, 153 (1990); C. K. Stover et al., *Nature* 351, 456 (1991); A. Aldovini and R. A. Young, *Nature* 351, 479 (1991); R. Schafer et al., J. *Immunol.* 149, 53 (1992); C. S. Hahn et al., *Proc. Natl. Acad. Sci.* (USA) 89, 2679 (1992). However, this approach may be undesirable for several reasons. Retroviral vectors, for example, have restrictions on the size and structure of polypeptides that can be expressed as fusion proteins while maintaining the ability of the recombinant virus to replicate (A.D. Miller, *Curr. Top. Microbiol. Immunol.* 158, 1 (1992). The effectiveness of vectors such as vaccinia for subsequent immunizations may be compromised by immune responses against vaccinia (E. L. Cooney et al., *Lancet* 337, 567 (1991)). Also, viral vectors and modified pathogens have inherent risks that may hinder their use in humans (R. R. Redfield et al., *New Engl. J. Med.* 316, 673 (1987); L. Mascola et al., *Arch. Intern. Med.* 149, 1569 (1989)). For example, in live vector approaches, highly immunogenic vectors also tend to be highly pathogenic.

Alternative gene delivery methods have also been explored. Benvenisty, N., and Reshef, L. (*PNAS* 83, 9551-9555, (1986)) showed that $CaCl_2$ precipitated DNA could be expressed in mice. Plasmid vectors have also been used to produce expression in mouse muscle cells (J. A. Wolff et al., *Science* 247, 1465 (1990); G. Ascadi et al., *Nature* 352, 815 (1991)). The plasmids were shown to be maintained episomally and did not replicate. Subsequently, persistent expression has been observed after i.m. injection in skeletal muscle of rats, fish and primates, and cardiac muscle of rats (H. Lin et al., *Circulation* 82, 2217 (1990); R. N. Kitsis et al., *Proc. Natl. Acad. Sci.* (USA) 88, 4138 (1991); E. Hansen et al.,

FEBS Lett. 290, 73 (1991); S. Jiao et al., *Hum. Gene Therapy* 3, 21 (1992); J. A. Wolff et al., *Human Mol. Genet.* 1, 363 (1992)). WO 90/11092 (4 Oct. 1990) reported the use of naked polynucleotides to vaccinate vertebrates.

Various routes of administration have been found to be suitable for vaccination using polynucleotide vaccines. Intramuscular administration is thought to be particularly desirable, given the proportionally large muscle mass and its direct accessibility through the skin. See U.S. Patent No. 5,580,859. Tang et al., (*Nature*, 356, 152-154 (1992)) disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice. Furth et al., (*Analytical Biochemistry*, 205, 365-368, (1992)) showed that a jet injector could be used to transfect skin, muscle, fat, and mammary tissues of living animals. WO 93/17706 describes a vaccination method wherein carrier particles are coated with a gene construct and then accelerated into a potential host. Intravenous injection of a DNA:cationic liposome complex in mice has also been reported (Zhu et al., *Science* 261, 209-211 (9 Jul. 1993); see also WO 93/24640). Methods for introducing nucleic acids have been reviewed (Friedman, T., *Science*, 244, 1275-1281 (1989)); see also Robinson et al., (Abstracts of Papers Presented at the 1992 meeting on Modern Approaches to New Vaccines, Including Prevention of AIDS, Cold Spring Harbor, p 92; *Vaccine* 11, 957 (1993)), where the intra-muscular, intra-venous, and intra-peritoneal administration of avian influenza DNA into chickens was alleged to have provided protection against lethal challenge.

Reports suggest that polynucleotide vaccination has provided effective protective immunity in various animal models. The immunization of mice against influenza by the injection of plasmids encoding influenza A hemagglutinin has been reported (Montgomery, D. L. et al., 1993, *Cell Biol.*, 12, pp. 777-783), or nucleoprotein (Montgomery, D. L. et al., supra; Ulmer, J. B. et al., 1993, *Science*, 259, pp. 1745-1749). The first use of DNA immunization for a herpes virus has been reported (Cox et al., 1993, *J. Virol.*, 67, pp. 5664-5667). Injection of a plasmid encoding bovine herpes virus 1 (BHV-1) glycoprotein g IV gave rise to anti-g IV antibodies in mice and calves. Upon intranasal challenge with BHV-1, immunized calves showed reduced symptoms and shed substantially less virus than controls. Wang et al., (*P.N.A.S.* USA 90, 4156-4160 (May, 1993)) reported on elicitation of immune responses in mice against HIV by intramuscular inoculation with a cloned, genomic (unspliced) HIV gene. However, the level of immune responses achieved was very low, and the system utilized portions of the mouse mammary tumor virus (MMTV) long terminal

repeat (LTR) promoter and portions of the simian virus 40 (SV40) promoter and terminator. SV40 is known to transform cells, possibly through integration into host cellular DNA. Thus, the system described by Wang et al., may be inappropriate for administration to humans.

It has been suggested to use calcium phosphate particles as agents for transfection of therapeutic polynucleotides in gene therapy. See U.S. Patent No. 5,460,831. DNA or RNA is attached to the particulate core and delivered to a target cell, resulting in expression of therapeutic proteins. However, this patent does not suggest the use of calcium phosphate particles as supports for DNA or RNA vaccines. To the contrary, this patent indicates that the stimulation of an immunological response during transfection is to be avoided. This patent also fails to suggest the use of calcium phosphate particles as controlled release matrices for genetic material.

With respect to RNA interference, there is also a need in the art to provide mechanisms to introduce nucleic acid material into a cell to inhibit the expression of a particular gene. Such therapy, often called RNA interference (RNAi) is useful in treating many of the above-described conditions. RNAi is a process by which double-stranded RNA triggers the degradation of a homologous messenger RNA (sharing sequence-specific homology to particular "target" mRNAs). Double-stranded RNAs (dsRNAs) that are complimentary to known mRNAs, are introduced into a cell to specifically block translation of that particular mRNA, thereby diminishing or abolishing gene expression.

To make the technique work in cultured mammalian cells, small interfering RNAs (siRNAs), which are double-stranded RNA and 21-25 nucleotides, are delivered into the cell. This is typically done using transfection reagents, which are solutions optimized for allowing DNA and RNA to be absorbed by cultured cells. The general goal for a delivery mechanism is to deliver the siRNA to the intracellular compartment of the cell (i.e. cytoplasm and / or nucleus), and other delivery mechanisms are needed.

RNAi differs from DNA immunization in many respects. For example, using the particles of the present invention to deliver DNA vaccines introduces genetic material that makes copies of itself and illicit an immune response, whereas RNA interference (RNAi) is used to block the translation of a particular gene. However, much of the discussion of DNA immunization is included in this application to provide examples of how the particle of this invention are able to deliver genetic material to a cell. The calcium phosphate particles provided by this invention are particularly useful in these efforts. Benefits are that it is a

good carrier, it is biocompatible, and it can be manufactured in a size that allows it to deliver material to a cell nucleus.

SUMMARY OF THE INVENTION

The present invention relates to novel calcium phosphate ("CAP") core particles, to methods of making them, and to methods of using them as vaccine adjuvants, as cores or carriers for biologically active material, and as controlled release matrices for biologically active material. More particularly, the invention relates to the core particles having a diameter between about 300 nm and about 4000 nm, more particularly between about 300 nm and about 1000 nm, and having a substantially spherical shape and a substantially smooth surface. In certain embodiments, the particles may be manufactured in a smaller size, for example, between about 150-500 nm, and more particularly, between about 200-400 nm. This size is particularly useful for the intracellular delivery of a large range of useful nucleotide sequences that are intended for the intracellular delivery of a large range of useful nucleotide sequences that are intended for use in RNA interference.

The present invention also relates to the novel calcium phosphate core particles having a material coated on the surface of the core particles, and/or dispersed or impregnated within the core particles, to methods of making them, and to methods of using them. Non-limiting examples of a suitable material to be at least partially coated on the surface of the core particle or impregnated therein include one or more polynucleotide material encoding immunogenic polypeptides, small inhibitory RNA, or therapeutic proteins or peptides.

The present invention also relates to combinations of this novel core particle having at least a partial coating of a surface modifying agent or a surface modifying agent impregnated therein or both. If one or more of the above-mentioned materials is at least partially coated on the particle, the material may be optionally attached to the particle by the surface modifying agent, which acts as a biological 'glue,' such as cellobiose or polyethylene glycol (PEG).

The invention also relates to methods of vaccinating patients in need thereof by administering the novel core particle in combination or in conjunction with a double stranded RNA for introduction into a cell to inhibit or silence expression of a particular gene. The RNA may be at least partially coated on the core particle and/or integrated therein, as described in more detail below. The core particles are sufficiently small to be easily transportable to various tissues throughout the body, particularly the intracellular

compartments of a cell (including cytoplasm and nucleus), and are biocompatible and biodegradable.

When administered as a polynucleotide vaccine or for RNA interference, the calcium phosphate in the core particles of the present invention biodegrades, releasing into the surrounding tissue polynucelotide material (DNA or RNA) coding for the desired sequence. Without wishing to be bound to any theory, it is believed that cells in the patient take up the DNA or RNA and express it. When used as a vaccine, the DNA or RNA is expressed as immunogenic proteins, which are then presented to B cells and T cells of the immune system, resulting in both a humoral and cell-mediated response similar to that obtained using live attenuated virus, but without the risks of pathogenicity and without the loss of immunogenicity associated with live virus. When the DNA or RNA is impregnated or dispersed within the calcium phosphate core particle, the gradual release of genetic material by the dissolution of the calcium phosphate matrix provides longer lasting immune responses than does administration of a conventional DNA or RNA vaccine.

In addition, while not wishing to be bound by any theory, it is believed that the presence of calcium phosphate core particles enhances the immune response to the antigenic proteins produced by the cells that take up and express the DNA or RNA, further multiplying the protective effect of the vaccine. The size of the core particles of the invention allows them to migrate through the body as the calcium phosphate gradually degrades, thereby transporting the DNA/RNA to different tissues in the body, and enlisting large numbers of different tissues at different locations in the production of antigenic proteins.

The present invention also relates to methods of preparing the novel calcium phosphate core particles described above, such as the core particles for use individually, the core particles having material at least partially coated on the surface, and the core particles having material impregnated therein.

The above discussed and many other features and attendant advantages of the present invention are detailed below.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B are photomicrographs of the calcium phosphate core particles according to one embodiment of the present invention.

Figure 2 presents a series of graphs showing the ELISA results for a calcium phosphate - tuberculosis antigen conjugate according to one embodiment of the present invention. Figure 2A shows ELISA results three weeks after primary vaccination. Figure 2B

shows ELISA results seven weeks after primary vaccination. Figure 2C shows ELISA results three months after primary vaccination. Figure 2D shows ELISA results five months after primary vaccination.

Figure 3 presents a series of graphs showing the ELISA results for a calcium phosphate - influenza antigen conjugate according to one embodiment of the present invention. Figure 3A shows ELISA results three weeks after primary vaccination. Figure 3B shows ELISA results eight weeks after primary vaccination. Figure 3C shows ELISA results ten weeks after primary vaccination.

Figure 4 presents a series of graphs showing ELISA results for a calcium phosphate - herpes simplex 2 antigen conjugate according to one embodiment of the present invention. Figure 4A shows ELISA results two weeks after primary vaccination. Figure 4B shows ELISA results four weeks after primary vaccination. Figure 4C shows ELISA results five weeks after primary vaccination.

Figure 5 is a graph showing ELISA and neutralization assay results for a calcium phosphate - herpes simplex 2 antigen conjugate according to one embodiment of the present invention.

Figure 6 is a graph showing ELISA results for a calcium phosphate - human immunodeficiency virus antigen conjugate according to one embodiment of the present invention.

Figure 7 presents a series of graphs showing ELISA results for a calcium phosphate-herpes simplex 2 antigen conjugate according to one embodiment of the present invention compared to the ELISA results for a calcium phosphate-herpes simplex 2 antigen conjugate made from Accurate CAP, which is produced by Superfos Biosector a/s. Figure 7A shows ELISA results with an IgG antibody titer. Figure 7B shows ELISA results with an IgG1 antibody titer. Figure 7C shows ELISA results with an IgG2a antibody titer.

Figure 8 presents a series of graphs showing the ELISA results for a calcium phosphate-Epstein-Barr virus antigen conjugate according to one embodiment of the present invention. Figure 8A shows ELISA results with an IgG antibody titer. Figure 8B shows ELISA results with an IgG2a antibody titer. Figure 8C shows ELISA results with an IgG1 antibody titer. Figure 8D shows ELISA results with an IgE antibody titer.

Figure 9 presents a series of graphs showing the ELISA results for a calcium phosphate-herpes simplex 2 antigen conjugate according to one embodiment of the present invention. Figure 9A shows ELISA results with an IgG antibody titer. Figure 9B shows

ELISA results with an IgG2a antibody titer. Figure 9C shows ELISA results with an IgG1 antibody titer. Figure 9D shows ELISA results with an IgE antibody titer.

Figure 10 presents a series of graphs showing the ELISA results for a calcium phosphate-tuberculosis antigen conjugate according to one embodiment of the present invention. Figure 10A shows ELISA results with an IgG antibody titer. Figure 10B shows ELISA results with an IgG2a antibody titer. Figure 10C shows ELISA results with an IgG1 antibody titer. Figure 10D shows ELISA results with an IgE antibody titer.

Figure 11 presents a series of graphs showing the ELISA results for a calcium phosphate-ovalbumin antigen conjugate according to one embodiment of the present invention. Figure 11A shows ELISA results with an IgG antibody titer. Figure 11B shows ELISA results with an IgG2a antibody titer. Figure 11C shows ELISA results with an IgG1 antibody titer. Figure 11D shows ELISA results with an IgE antibody titer.

Figure 12 is a schematic drawing showing a calcium phosphate core particle (4) both coated with antigenic material or natural immunoenhancing factor (8) and having antigenic material or natural immunoenhancing factor (8) impregnated therein.

Figure 13 is a series of schematic drawings showing embodiments having a calcium phosphate core particle (4) coated with material (6), such as antigenic material, natural immunoenhancing factors, polynucleotide material encoding immunogenic polypeptides, or therapeutic proteins or peptides, or having material (6) impregnated therein. Figure 13A shows a core particle coated directly with material (6). Figure 13B shows a core particle (4) coated with surface modifying agent (2), such as polyethylene glycol or cellobiose, and a having a material (6) adhered to the surface modifying agent (2). Figure 13C shows a calcium phosphate core particle (4) having a surface modifying agent (2), such as polyethylene glycol or cellobiose incorporated therein and having a material (6) at least partially coating core particle (4).

Figure 14 is a schematic drawing showing a calcium phosphate core particle (4) having both a surface modifying agent (2), such as polyethylene glycol or cellobiose and a material (6), such as antigenic material, natural immunoenhancing factors, polynucleotide material encoding immunogenic polypeptides, or therapeutic proteins or peptides incorporated therein.

DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention relates to novel calcium phosphate core particles, to methods of making them, and to methods of using the core particles as vaccine adjuvants, as cores or

carriers for biologically active material, and as controlled release matrices for biologically active material. The present invention also relates to the novel calcium phosphate core particles having a material at least partially coated on the surface of the core particles, or dispersed or impregnated within the core particles, to methods of making them, and to methods of using them. The core particles of the present invention may optionally have at least a partial coating of a surface modifying agent, which may help adhere the above-mentioned material to the core particle, or may have a surface modifying agent impregnating the particle, or both.

One embodiment of the present invention relates to calcium phosphate core particles suitable for adjuvanting vaccines, the particles being administerable in their uncoated state. The core particles are also suitable for use as supports for microbial antigenic material or natural immunoenhancing factor (as cores to be at least partially coated with microbial antigenic material or natural immunoenhancing factor) and for providing a controlled or sustained release matrix for biologically active molecules. As used herein, the term "antigenic material" or "antigen" means an immunogenic antigen product obtained from a bacteria, virus, or fungus, and containing one or more antigenic determinants. Examples of antigenic material as this term is used herein include one or more portions of the protein coat, protein core, or functional proteins and peptides of a virus, such as Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), human papilloma virus (HPV), herpes simplex virus (HSV), pox virus, influenza, or other virii, or immunogenic proteins obtained from bacteria, such as tuberculosis (TB), staphylococcal, streptococcal, clostridium, pseudomonas, or coliform bacterial antigens, or fungi, such as candida and other saccharomyces. The binding activity of calcium phosphate core particles allows a high loading capacity for these different types of proteins.

Another embodiment of the present invention relates to calcium phosphate core particles modified to function as polynucleotide vaccines, having DNA or RNA encoding immunogenic polypeptides at least partially coated on the surface of the core particles or at least partially impregnated therein. Exemplary polynucleotides include those encoding immunogenic epitopes for influenza, malaria, colon cancer cells, hepatitis B, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), cutaneous T cell lymphoma, herpes simplex, tick born encephalitis, rabies, rotavirus, tuberculosis, Epstein-Barr virus, human papilloma virus, and hepatomavirus. When administered to a patient, the core particle biodegrades and the DNA or RNA is taken up and expressed by the cells and

translated to produce one or more immunogenic polypeptides that are recognized by the immune system.

Another embodiment of the present invention relates to calcium phosphate core particles modified to function as delivery mechanism for small inhibitory RNA (siRNA) for RNA interference to inhibit expression of a particular gene.

I. CORE PARTICLES

The calcium phosphate core particles of the present invention have an average particle size between about 300 nm and about 4000 nm, more particularly, between about 300 nm and about 2000 nm. An average particle size of between about 300 nm and 1000 nm is sufficient and desirable. In other embodiments, the particles may be sized between 100-500 nm, and more particularly, between 200-400 nm. This size may be beneficial for use with the RNA interference embodiment of the invention in order to deliver the material to the intracellular compartments of a cell (i.e. cytoplasm and / or nucleus). The core particles of the present invention have a morphology that is generally and substantially spherical in shape and a surface that is substantially smooth.

The term "substantially smooth" is used herein to mean essentially no surface features or irregularities having a size of 100 nm or larger. The core particles may be faceted or angular and still fall within this definition, as long as the facets do not contain many surface irregularities of the type described above. The term "substantially spherical" is used herein to refer to particles that are substantially round or oval in shape, and includes particles that are unfaceted and smooth, or that have very few facets, as well as particles that are polyhedral having several or numerous facets. Substantially smooth, substantially spherical particles according to the invention are visible in scanning electron micrographs and shown in Figures 1A and 1B.

The following table provides a comparison between the calcium phosphate core particles of the present invention and calcium phosphate particles manufactured by Superfos Biosector a/s, referred to as "Accurate CAP" in Figures 7A-C. The table shows that the calcium phosphate core particles of the present invention are small, smooth and ovoid, whereas Superfos Accurate CAP particles are large, jagged and crystalline.

	BioSante Pharmaceuticals, Inc. CAP	**Superfos Biosector a/s CAP**

pH	6.2 – 6.8	6.49
Size	< 1000 nm	> 3000 nm
Morphology	Smooth ovoid shape	Jagged crystalline shape
Antibody response:		
IgG	See Figure 7A	See Figure 7A
IgG1	See Figure 7B	See Figure 7B
IgG2a	See Figure 7C	See Figure 7C

The calcium phosphate core particles of the present invention are typically prepared as a suspension in aqueous medium by reacting a soluble calcium salt with a soluble phosphate salt, and more particularly, by reacting calcium chloride with sodium phosphate under aseptic conditions. Initially, an aqueous solution of calcium chloride having a concentration between about 5 mM and about 100 mM is combined by mixing with an aqueous solution of a suitable distilled water-based solution of sodium citrate, having a concentration between about 5 mM and about 100 mM. The presence of sodium citrate contributes to the formation of an electrostatic layer around the core particle, which helps to stabilize the attractive and repulsive forces between the core particles, resulting in physically stable calcium phosphate core particles.

An aqueous solution of dibasic sodium phosphate having a concentration between about 5 mM and about 100 mM is then mixed with the calcium chloride/sodium citrate solution. Turbidity generally forms immediately, indicating the formation of calcium phosphate core particles. Mixing is generally continued for at least about 48 hours, or until a suitable core particle size has been obtained, as determined by sampling the suspension and measuring the core particle size using known methods. The core particles may be optionally stored and allowed to equilibrate for about seven days at room temperature to achieve stability in size and pH prior to further use.

In one embodiment, the calcium phosphate core particles of the present invention can be used without further modification as vaccine adjuvants. For instance, the core particles may be uncoated and can be administered in a dosage of about 1 μg to about 1000 μg per kilogram of total body weight in conjunction with killed, attenuated, or live vaccines, with decoy viruses, or with core particles at least partially coated with microbial antigenic

material, such as those described above. The killed, live, or attenuated vaccines, decoy viruses, or antigen-coated core particles may be administered in the same solution as, or in a different solution from, that of the uncoated particles.

In another embodiment, the core particles of the present invention can also be at least partially coated with material, wherein the material is disposed on the surface of the core particle and optionally held in place by a surface modifying agent sufficient to bind the material to the core particle without denaturing the material. Non-limiting examples of the material disposed on the surface of the core particles include antigenic material or natural immunoenhancing factor, polynucleotide material, or therapeutic proteins or peptides.

Surface modifying agents suitable for use in the present invention include substances that provide a threshold surface energy to the core particle sufficient to bind material to the surface of the core particle, without denaturing the material. Example of suitable surface modifying agents include those described in U.S. Patent Nos. 5,460,830, 5,462,751, 5,460,831, and 5,219,577, the entire contents of each of which are incorporated herein by reference. Non-limiting examples of suitable surface modifying agents may include basic or modified sugars, such as cellobiose, or oligonucleotides, which are all described in U.S. Patent No. 5,219,577. Suitable surface modifying agents also include carbohydrates, carbohydrate derivatives, and other macromolecules with carbohydrate-like components characterized by the abundance of -OH side groups, as described, for example, in U.S. Patent No. 5,460,830. Polyethylene glycol (PEG) is a particularly suitable surface modifying agent. Other modifyinag agents may be a polyadenosine sequence (the process is referred to as "polyadenylation"). (For example, such as an AAAA nucleotide sequence may either be physically conjugated (i.e. either covalently or non-covalently) with the calcium phosphate particles.

The core particles may be at least partially coated by preparing a stock solution of a surface modifying agent, such as cellobiose or PEG (e.g., around 292 mM) and adding the stock solution to a suspension of calcium phosphate core particles at a ratio of about 1 mL of stock solution to about 20 mL of particle suspension. The mixture can be swirled and allowed to stand overnight to form at least partially coated core particles. The at least partially coated core particles are administerable alone or in conjunction with one or more of the materials described below. Generally, this procedure will result in substantially complete coating of the particles, although some partially coated or uncoated particles may be present.

II. ANTIGENIC MATERIAL OR NATURAL IMMUNOENHANCING FACTOR

In one embodiment, the uncoated core particles or the core particles at least partially coated with surface modifying agent are then contacted with antigenic material or natural immunoenhancing factor, to produce particles having antigenic material or natural immunoenhancing factor at least partially coating the core particle. Figure 12 is a schematic drawing of the particles of this embodiment, illustrating antigenic material or natural immunoenhancing factor (8) both coating the core particle (4) and incorporated within the core particle (4) (as will be discussed below). Antigen purified from viral coat or capsule proteins, or from cell surfaces of bacteria or fungi, can be obtained or purified using methods that are known in the art, or can be obtained commercially. For example, viral particles are obtained by infecting transforming host cell lines with the virus, and after a suitable incubation period, centrifuging the cell suspension and sonicating the resulting suspension at high power for several minutes to break open the cells, and again centrifuging the broken cell suspension. The supernatant containing virus can then be stored for further processing and protein purification using techniques familiar to those skilled in the art. Bacterial and fungal cell membrane antigens can be obtained by culturing and lysing the desired organisms and separating the desired antigenic protein fractions using techniques known in the art.

The antigen-coated particles of the invention are not produced by methods requiring the denaturing of the protein coating of a viral particle, removal of the core viral genetic material, and renaturing of the protein coating around a substitute core. Instead, the antigen-coated particles of the invention result from attachment of individual portions of protein coating to a calcium phosphate core. As a result, the particles of the invention are not believed to function as "decoy viruses" per se, as described in several of the patents cited above.

Instead, the particles of the invention can be more potent immunogenically than can a decoy virus, since only immunogenic portions of proteins need be attached to the particles. This increases the likelihood, for a given concentration of particles, that an antigenic epitope on the particle will elicit an immune response. In addition, the particles of the invention can be used to provide a broader spectrum of protection, since immunogenic material from several different pathogens can be attached to the surface of a single particle, or to the surfaces of different particles administered substantially simultaneously. These advantages are not obtained with the viral decoy particles described in the above-identified patents.

In addition to an antigen coating or in the alternative, the calcium phosphate core particles of the present invention can be prepared as controlled release particles for the

sustained release of antigenic material or natural immunoenhancing factor over time, wherein the antigenic material or natural immunoenhancing factor (8) is incorporated into the structure of the core particle (4), shown in Figure 12. This is done by mixing the aqueous calcium chloride solution with the antigenic material or natural immunoenhancing factor to be incorporated prior to combining and mixing with either the sodium citrate or dibasic sodium phosphate solutions, to co-crystallize the calcium phosphate core particles with the antigenic material or natural immunoenhancing factor. The antigenic material may consist of an immunogenic antigen product obtained from a bacteria, virus, or fungus, and containing one or more antigenic determinants, as described in detail above. The natural immunoenhancing factor may consist of proteins or peptides that function as a natural adjuvant, such as interleukins, particularly interleukin-2 and interleukin-12, also described in detail above.

III. NUCLEOTIDE MATERIAL

If polynucleotide material is coated onto and/or incorporated within the core particle, the particles function as a controlled release matrix for the DNA or RNA. The DNA or RNA that is at least partially coated onto or incorporated within the core particles may be selected from a wide variety of DNA or RNA sequences that encode epitopes of one or more immunogenic polypeptides, and thus can be used as the active ingredient in a DNA or RNA vaccine. Antisense fragments may also be used. Exemplary polynucleotides include those encoding immunogenic epitopes for influenza, malaria, colon cancer cells, hepatitis B, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), cutaneous T cell lymphoma, herpes simplex, tick born encephalitis, rabies, rotavirus, tuberculosis, Epstein-Barr virus, human papilloma virus, and hepatomavirus.

If the particles are used for RNA interference, it should be noted that the small inhibitory RNA that is delivered to the cell may be intended to deliver blocking sequences to inhibit expression of particular genes (generally, without rendering the siRNA immunogenic).

The nucleic acid material can be naked or inserted into a plasmid vector. Suitable plasmids are known to those skilled in the art, and typically include pcDNA3 (Invitrogen), pCI (Promega) and PBR231. It may be desirable that the plasmid or naked DNA express cytomegalovirus (CMV) intermediate-early promoter, or bovine growth hormone polyadenylation sequence. A large number of expression vectors can be constructed by incorporating a cDNA sequence encoding an immunogenic polypeptide into a plasmid vector.

The DNA or RNA segments may be prepared, inserted into vectors, and the vectors cloned according to known procedures, such as the procedures described in Maniatis, et al., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, New York, 1.0 - 19.0 (1989). Gene segments are also available commercially from a number of different suppliers, and inserted into commercially available plasmids. When the sequence of a candidate protein is known, a coding sequence of the polynucleotide can typically be inferred and the corresponding gene segment prepared and isolated.

The polynucleotide sequence may be fused with other sequences in the vector, such as human tissue plasminogen activator leader peptide. The vectors can also include bacterial DNA or naked DNA surrounding the gene for the pathogenic antigen as a foreign sequence motif, increasing the immune response to that gene. See Y. Sato et al., *Science* 273:352-354 (1996); G. J. Weiner et al., *PNAS* 94(20): 10833-7 (1997). Moreover, the plasmid may also include other genetic adjuvants, such as genes coding for cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukins, to further multiply the immune response.

To form core particles having at least a partial coating of polynucleotide material, the at least partially coated core particles described above are contacted with polynucleotide material, i.e., DNA or RNA coding for one or more antigens expressed by organisms to be vaccinated against. When the core particles are coated, the DNA or RNA material is attached to the surface of the coating as described in U.S. Patent No. 5,460,831. Figure 13A shows a schematic drawing of the particles of this embodiment, with material (6), such as polynucelotide material coating the core particle (4).

In addition to a polynucleotide coating or in the alternative, polynucleotide material may be incorporated into the structure of the core particle. For example, the DNA or RNA coding for an epitope expressed on a viral protein coat or capsule can be mixed with a solution of calcium chloride, which can then be mixed with, e.g., a buffer, such as a sodium citrate solution, and a solution of dibasic sodium phosphate. The resulting particles will have the DNA or RNA dispersed or impregnated therein. A vector containing the DNA or RNA may also be added with one or more of the reactants forming the core particle, as described above. For example, a plasmid or other vector containing immunogen-encoding DNA or RNA or naked DNA can be mixed with the calcium chloride solution, so that the calcium phosphate biodegradable matrix forms around the plasmid or naked DNA, which becomes embedded in and/or on the core particle.

The impregnated or coated core particle fragments can be separated from the production mixture and stored for further use. Storage can be by any conventional methods for storing gene segments or antisense fragments. For example, the core particles may be lyophilized or stored as a suspension in a compatible solution.

A typical polynucleotide vaccine produced according to the present invention contains about 0.5 to 500 micrograms of DNA or RNA material. When administered, the core particles are combined with a pharmaceutically acceptable carrier solution or other excipient. The dose will vary with the route of administration, the frequency of treatment, and other patient characteristics. Typical vaccination dosages include from about 0.1 mL to 2 mL of a vaccine containing about 0.5 to 500 micrograms of DNA or RNA material. Because the core particle supporting the DNA or RNA is biodegradable calcium phosphate, DNA or RNA that may impregnated therein is slowly released over time as the particles dissolve under physiological conditions. DNA or RNA released from the dissolving material is taken up and expressed by cells, and translated to produce one or more immunogenic polypeptides that are recognized by the humoral and cell-mediated immune system in the same manner as if the antigen had been vaccinated conventionally, but without the risks associated with the administration of live attenuated or killed virus. Moreover, the presence of calcium phosphate particles that have not completely dissolved serves an adjuvanting function for the DNA or RNA vaccine by enhancing the efficacy of the immunogenic protein or proteins expressed by the cells taking up the DNA or RNA.

It is also contemplated that the particles of the present invention may be used in RNA-based therapeutics, such as those being developed by Sirna Therapeutics™, headquartered in Boulder, Colorado and Alnylam™ Pharmaceuticals, headquartered in Cambridge, Massachusetts.

RNA interference (RNAi) is the introduction of double-stranded RNA into a cell to inhibit the expression of a gene. Small inhibitory RNA (siRNA) is a short sequence of RNA which can be used to silence gene expression. Currently, Sirna™ and Alnylam™, among other organizations, are working on the development of a new class of drugs, capable of silencing specific disease-causing genes, using siRNA and RNAi.

It is contemplated that siRNA and/or other nucleotide materials necessary for RNAi may be delivered by the CAP particles of the present invention. It may be coated onto and/or incorporated within the core particle. The core particles may be administered alone or in combination with a pharmaceutically acceptable carrier. Administration may take place by

any route which delivers the particles across cell membranes, including, without limitation, orally, nasally, ocularly, subcutaneously and vaginally. Non-limiting examples of diseases which may be treated by the present invention include macular degeneration, metabolic diseases such as diabetes and high cholesterol, viral diseases such as hepatitis C, autoimmune diseases, infectious diseases, inflammatory diseases and cancer. The particles may be delivered orally, intranasally, intratracheally, intrapulmonary (e.g. via aerosolization of particles containing biological payload), bucally (e.g., via the tissues of the mouth), intraocularly (e.g., via an eye drop or an ointment), intratumorally, subcutaneously, or vaginally. The various embodiments of the invention can be more clearly understood by reference to the following nonlimiting examples.

EXAMPLE 1

A 12.5 mM solution of $CaCl_2$ is prepared by mixing 1.8378 g of $CaCl_2$ into 800 mL of sterile GDP water under aseptic conditions until completely dissolved, and the solution diluted to 1 L and filtered. A 15.625 mM solution of sodium citrate was prepared by dissolving 0.919 g of sodium citrate into 200 mL of sterile GDP water with mixing using aseptic techniques and filtered. A 12.5 mM solution of dibasic sodium phosphate was prepared by dissolving 1.775 g sodium phosphate into 1 L of sterile GDP water with mixing using aseptic techniques and filtered. All solutions were stored at room temperature.

The calcium chloride solution was combined with the sodium citrate solution and thoroughly mixed. Subsequently, the sodium phosphate solution was added with mixing. Turbidity appeared immediately as particles began to form. The suspension was allowed to mix for several minutes and was sampled for endotoxin testing using aseptic technique. Mixing was continued for about 48 hours under a laminar flow hood. Following mixing, the particles were sonicated on a high power setting for about 30 minutes at room temperature, tested for endotoxin concentration and pH and characterized as to particle size with a Coulter N4Plus Submicron Particle Sizer. Photomicrographs of particles prepared in this way are shown in Figures 1A and 1B. Following preparation the particles were allowed to equilibrate for approximately seven days before use.

EXAMPLE 2

An HSV-2 protein solution and an Epstein-Barr virus (EBV) protein solution were purified from ATCC VR-540 (infected tissue culture fluid and cell lysate). The viral suspension was contacted with a lysis buffer (1% IGEPAL CA-630 for HSV-2 and 1% Triton x 100 for EBV, 10 mM NaCl, 10 mM Tris-HCL, and 1.5 mM $MgCl_2$), vortexed for 1 minute,

incubated on ice for 30 minutes, and centrifuged at 1400 rpm for 2 hours at 4° C. The resulting supernatant was then contacted with a second lysis buffer (1 mM PMSF, 1% IGEPAL CA-630 for HSV-2 and 1% Triton x 100 for EBV, 100 mM NaCl, 100 mM Tris-HCL, and 3 mM $MgCl_2$), incubated on ice for 30 minutes, and centrifuged at 1400 rpm for 2 hours. The supernatant was then dialyzed against 2L of 0.9% saline overnight, lyophilized and resuspended in 1 mL PBS.

EXAMPLE 3

25 mL of 12.5 mM calcium chloride, 5 mL of 15.625 mM sodium citrate, and 25 mL of 12.5 mM dibasic sodium phosphate solutions were prepared as described in Example 1. The calcium chloride solution was mixed with 1.3 mL of purified HSV-2 protein prepared according to Example 2, which mixing was continued for about 1 minute. 5 mL of sodium citrate was added to the calcium chloride/HSV-2 mixture and allowed to mix for 1 minute. 25 mL of dibasic sodium phosphate was added to the mixture, which immediately becomes turbid, indicating the formation of particles. The mixture is stirred at a moderate speed for 48 to 96 hours, or until the particle size is less than 1000 nm, as determined using a Coulter N4Plus Submicron Particle Sizer, and sonicated. After preparation the particles were stored for approximately seven days before use to allow equilibration of particles to reach size stability.

The resulting particles, containing HSV-2 protein dispersed therein, can be administered as a sustained release vaccine in dosages of about 1 μg to about 250 μg per kg of body weight.

EXAMPLE 4

A suspension of calcium phosphate particles is prepared following the procedures of Example 1, and the particle size and presence of any endotoxin determined. Cellobiose glue is applied to the particles by suspending them in a solution of 292 mM cellobiose stock added to the suspension of calcium phosphate particles at a ratio of 1 mL of cellobiose solution to 20 mL of particle suspension. The mixture is gently mixed and allowed to stand overnight. The at least partially coated particles are then contacted with a solution of cell surface proteins of tuberculosis bacilli (provided by the Morehouse School of Medicine), and co-incubated at room temperature or at 4°C (as desired).

The resulting particles were characterized by measuring their particle size using a Coulter N4Plus Submicron Particle Sizer, and had an average diameter of <1000 nm.

The efficacy of the particles was tested as follows. Six mice each (for a total of 30 mice) were injected with solutions containing antigen only, calcium phosphate particles only, antigen + Imject (an alum based adjuvant), washed (with PBS three times, with each washing followed by centrifugation at 4500 rpm for 15 minutes at 4°C) calcium phosphate + antigen, and unwashed calcium phosphate + antigen. The total injection volume for each immunization per mouse was 100 μL.

For the injection of antigen only, the first immunization contained 10 μg of antigen administered intraperitoneally (i.p.), and the second injection contained 10 μg of antigen ("TB only").

For the injection of calcium phosphate particles only, 0.46 mg of this concentrated solution of particles were administered per mouse ("CAP only").

For the injection of antigen + Imject, 10 μg of antigen and 50 mg of alum-based Imject were administered per mouse, i.p. ("TB + Imject").

For the washed calcium phosphate particles + antigen, 10 μg of antigen was coated onto 1.0 mg of calcium phosphate particles, and after washing with PBS, centrifugation, precipitation, and resuspension (three times) was injected i.p. ("Washed CAP-TB").

For the unwashed calcium phosphate particles + antigen, 10 μg of antigen was coated on 1.0 mg calcium phosphate particles and administered i.p., without further treatment ("Unwashed CAP-TB").

Blood samples were collected approximately three weeks later and subjected to ELISA to measure serum TB-specific antibody. Booster immunizations were given at a concentration of 1 μg approximately 14 days after primary immunization, and blood samples collected and subjected to ELISA about one per month after the booster immunization and about every two months thereafter. The results are provided in Figure 2.

EXAMPLE 5

25 mL of 12.5 mM calcium chloride, 5 mL of 15.625 mM sodium citrate, and 25 mL of 12.5 mM sodium phosphate dibasic solutions are prepared as described in Example 1. The calcium chloride solution is mixed with of DNA encoding obtained an immunogenic polypeptide of a disease-causing pathogen, prepared according to techniques familiar to those skilled in the art. 5 mL of sodium citrate is added to the calcium chloride/DNA mixture and allowed to mix for 1 minute. 25 mL of dibasic sodium phosphate is added to the mixture, which will immediately become turbid, indicating the formation of particles. The mixture is

stirred at a moderate speed for 48 to 96 hours, or until the particle size is less than 1000 nm, as determined using a Coulter N4Plus Submicron Particle Sizer, and sonicated.

The resulting particles, containing DNA encoding an immunogenic polypeptide dispersed therein, can be administered as a sustained release DNA vaccine in dosages of about 1 μg to about 250 μg per kg of body weight.

EXAMPLE 6

Procedures similar to those described above in Examples 4 and 5 were followed, to prepare and evaluate a cellobiose-coated calcium phosphate particle suspension combined with immunogenic herpes simplex 2 viral protein. The protein is prepared from ATTC VR-540 using the protein purification procedures described in Example 2.

50 mL of calcium phosphate suspension prepared as described in Example 1 and coated with cellobiose glue as described in Example 3 were centrifuged at 4500 rpm for 15 minutes at 25 °C, and the supernatant discharged. The pellet was resuspended in 2.5 mL of spent buffer from the production of the calcium phosphate particles, so that the calcium phosphate concentration was increased 20 fold. The concentrated calcium phosphate was divided into 1 mL aliquots. 1 mL of HSV protein was added to the concentrated calcium phosphate suspension and rotated for 1 hour at 4 °C. One aliquot of this suspension was not washed (UWCCH). The other was washed with PBS (and centrifuged at 4500 rpm for 15 minutes at 4°C) three times and resuspended in 2 mL PBS (WCCH solution).

50 mL of calcium phosphate co-crystallized with HSV-2 suspension as described in Example 2 were centrifuged at 4500 rpm for 15 minutes at 25 °C, and the pellet resuspended in 2.5 mL of spent calcium phosphate buffer. 1 mL of this concentrated calcium phosphate-HSV-2 particle solution was mixed with 1 mL of HSV-2 protein and rotated for 1 hour at 4 °C. This solution was washed with PBS (and centrifuged at 4500 rpm for 15 minutes at 4°C) three times and resuspended in 2 mL PBS (WCHCH solution).

1 mL of Imject (alum adjuvant) was mixed with 1 mL of HSV-2 protein solution (IH).

1 mL of HSV-2 protein solution was mixed with 1 mL of PBS (HIV).

Protein assays were conducted on wash supernatants to determine the percent binding of HSV-2 to the calcium phosphate complexes. Binding was generally > 20%.

Immunization testing was carried out as described in Example 3 above, except that a primary immunization and two booster immunizations were administered approximately one

month and three weeks apart, respectively. All immunizations were administered intraperitoneally. The amounts administered are provided in the Table below.

HSV-2 Only Primary Immunization (HSV)	52.5 μg antigen
HSV-2 Only Second Immunization (HSV)	89 μg antigen
HSV-2 Only Third Immunization (HSV)	129 μg antigen
HSV-2 + Imject (IH)	52.5 μg HSV-2 antigen 50 mg Imject
Washed Calcium Phosphate + HSV-2 (WCCH)	42.9 μg HSV-2 antigen 0.46 mg Calcium Phosphate
Unwashed Calcium Phosphate + HSV-2 (UWCCH)	52.5 μg HSV-2 antigen 0.46 mg Calcium Phosphate
Washed Calcium Phosphate Co-crystallized with HSV-2 + HSV-2 (WCHCH)	29.1 μg HSV-2

Blood was collected and analyzed by ELISA about one month after the primary injection and about 14 days after each booster injection and again two months after the third ELISA. The results are presented in Figures 4 and 5.

Immunized mice were challenged intravaginally with 10^2 PFU of HSV-2 at 30 days after primary immunization in accordance with the methods discussed in Dr. Rouse et al., 1997, to test resistance. Since the stage of estrus can affect susceptibility to HSV infection, mice were given progesterone injection to synchronize the estrus cycle prior to challenge with HSV-2. The results are presented below.

	No. of mice survived/ No. of mice challenged	Clinic severity at week Post challenge		
		1	2	3
Control	3/5	1	1.3	2
Alum + HSV-2	5/5	0	0	0
CAP + HSV-2	5/5	0	0	0

Note: The mice were observed everyday for vaginal inflammation. Clinic severity was graded as follows: 0. No inflamation; 1. Mild inlflamation; 2. Moderate swelling and redness; 3. Severe inflamation; 4. Paralysis; and 5. Death.

EXAMPLE 7

The procedures described above were carried out using HIV-1 antigen prepared from 10-119-000 (Advanced Biotechnologies, Inc.) using the protein purification procedures described in Example 2. Solutions of antigen alone (6.9 μg HIV per mouse), washed calcium phosphate/ cellobiose/antigen particles (9.5 μg HIV per mouse), and antigen with Imject adjuvant (6.9 μg HIV per mouse) were each administered to 6 mice as described above, and anti-HIV antibody titer was evaluated by ELISA two weeks after primary infection. The results are presented in Figure 6.

EXAMPLE 8

Four different antigens were combined with CAP to study its effectiveness as an adjuvant. These four antigens included Ovalbumin (Ova), Tuberculosis (TB), HSV-2 and EBV.

The Ovalbumin and Tuberculosis coated particles were prepared following the procedures of examples 1 and 4. The cellobiose coated CAP was mixed for one hour with 0.5 mg of Ovalbumin or Tuberculosis antigen. The samples were then washed three times (centrifuged at 4500 rpm for 15 minutes at 4° C) with PBS. 1 mL of Imject-alum adjuvant was mixed with the same amount of Ovalbumin and Tuberculosis. A solution of antigen alone was prepared by mixing 0.5 mg of Ovalbumin and Tuberculosis antigen with 1 mL of PBS respectively.

The HSV-2 CAP and EBV CAP were prepared by co-crystallizing the viral protein with the CAP similar to the procedure described in Example 3. The resulting CAP with either HSV-2 or EBV dispersed therein was then subsequently treated with cellobiose and the surface coated with antigen as described above.

Six mice each were immunized by i.p. injection with one of the following antigens: Ova alone, Ova+Alum, Ova + CAP, TB alone, TB + Alum, TB + CAP, HSV-2 alone, HSV-2 + Alum, HSV-2 + CAP, EBV alone, EBV + Alum, EBV + CAP and CAP alone. The concentration of CAP to Alum is 1:100 for the OVA and TB vaccine constructs. The concentration of CAP to Alum is equivalent for HSV-2 and EBV vaccine constructs. All mice were immunized with a primary injection and given two or three booster injections at two-week intervals. Blood was collected and IgG, IgGI, IgG2a, and IgE antibody titers in immunized mice were measured by ELISA. The results are presented in Figures 8-11, which show different levels of antibodies between the different groups of immunized mice.

EXAMPLE 9

A suspension of calcium phosphate particles is prepared following the procedures of Example 1, and the particle size and presence of any endotoxin determined. Cellobiose glue is applied to the particles by suspending them in a solution of 292 mM cellobiose stock added to the suspension of calcium phosphate particles at a ratio of 1 mL of cellobiose solution to 20 mL of particle suspension. The mixture is gently mixed and allowed to stand overnight. A cDNA encoding an immunogenic polypeptide of a disease-causing pathogen is inserted into a pcDNA3 plasmid according to techniques familiar to those in the art. The coated particles are then contacted with a solution of plasmid DNA, and co-incubated at room temperature or 40°C.

EXAMPLE 10

Calcium phosphate core particles of the present invention, (CAP), were tested in comparison to calcium phosphate particles manufactured by Superfos Biosector a/s, referred to as "Accurate CAP," to study the effectiveness of the CAP particles of the present invention as an adjuvant.

HSV-2 CAP was prepared by co-crystallizing the viral protein with the CAP similar to the procedure described in Example 3. The resulting CAP with HSV-2 dispersed therein was then subsequently treated with cellobiose and the surface coated with antigen as described above.

Five mice each were immunized by i.p. injection with one of the following antigens: HSV-2+CAP or HSV-2+Accurate CAP. The mice were immunized with a primary injection and given two or three booster injections at two-week intervals. Blood was collected and IgG, IgGI, and IgG2a antibody titers in immunized mice were measured by ELISA. The results are presented in Figures 7A-C, which show different levels of antibodies between the different groups of immunized mice. Of particular importance is the result shown in Figure 7C. Figure 7C shows that the IgG2a antibody titer for the CAP particles of the present invention triggered a strong IgG2a response.

The procedures described above and exemplified above can be modified by those having skill in the art to yield other embodiments of the invention. For example, the material to be dispersed throughout the particle can be co-crystallized and impregnated within the particle as described above, and the resulting particles can be coated with the same or different material, using the coating methods described above. The core particles may also

have a partial coating of one or a mixture of surface modifying agents described above to help adhere material coating the particle to the surface thereof.

The present invention has been described above with respect to certain specific embodiments thereof, however it will be apparent that many modifications, variations, and equivalents thereof are also within the scope of the invention.

What is claimed is:

1. A particle comprising calcium phosphate having a substantially spherical shape and a substantially smooth surface, comprising a sequence of small inhibitory RNA material at least partially coating the particle, impregnating the particle, or both.

2. The particle of claim 1, further comprising a surface modifying agent at least partially coating the particle or impregnating the particle or both.

3. The particle of claim 2, wherein the surface modifying agent is at least partially disposed between the surface of the particle and the polynucleotide material.

4. The particle of claim 2, wherein the surface modifying agent comprises a basic or modified sugar, cellobiose, or an oligonucleotide.

5. The particle of claim 2, wherein the surface modifying agent comprises a carbohydrate, a carbohydrate derivative, a macromolecule with carbohydrate-like components characterized by the abundance of -OH groups, or a polyadenosine sequence.

6. The particle of claim 2, wherein the surface modifying agent comprises polyethylene glycol.

7. The particle of claim 1, wherein the small inhibitory RNA sequence is complementary in sequence to a specific mRNA coding sequence and is adapted to bind and block the translation process of the mRNA.

8. The particle of claim 7, wherein the RNA sequence is complementary to an mRNA sequence that encodes one or more proteins or peptides normally produced by virii bacteria, or parasites, or cells causing influenza, malaria, colon cancer, hepatitis 3, human immunodeficiency virus, (HIV), simian immunodeficiency virus (SIV), cutaneous T cell lymphoma, herpes simplex, tick born encephalitis, rabies, rotavirus, tuberculosis, Epstein-Barr virus, human papilloma virus, hepatomavirus, or cancer-causing cells.

9. The particle of claim 8, wherein the RNA or DNA sequences are antisense fragments.

10. The particle of claim 8, wherein the RNA or DNA sequence is inserted into a plasmid vector.

11. The particle of claim 10, wherein the plasmid vector is selected from the group consisting of including pcDNA3 (Invitogen), pcI (Promega) and PBR231.

12. The particle of claim 10, comprising DNA or RNA that is fused with other DNA or RNA sequences in the form of plasmid vector or naked DNA.

13. The particle of claim 12, wherein the RNA sequences have the potential to block production of infectious material, cancer causing genes, and cell-derived products such as enzymes, hormones and growth factors.

certain growth factors.

14. The particle of claim 1, wherein the RNA sequence is intended to inhibit the expression of a gene.

15. The particle of claim 14, wherein the RNA is small inhibitory RNA (siRNA).

16. A method of preparing a composition for treatment to inhibit the expression of a desired gene, comprising:

(a) providing a sequence of oligonucleotide material that is intended to inhibit expression of the desired gene;

(b) associating the oligonucleotide material with a calcium phosphate particle by

(i) mixing an aqueous solution of calcium chloride with an aqueous solution of sodium citrate to form a mixture,

(ii) adding an aqueous solution a sodium phosphate to the mixture to form a solution,

(iii) stirring the solution until particles of the desired size and comprising calcium phosphate are obtained; and

(iv) contacting the particles with a solution of oligonucleotide material to form particles that are at least partially coated with the oligonucleotide material.

17. The method of claim 16, further comprising:

prior to contacting the particles with the oligonucleotide material, adding a surface modifying agent to a suspension of calcium phosphate particles and allowing the suspension to stand for sufficient time for the surface modifying agent to cover at least a portion of the particles to form at least partially coated particles.

18. The method of claim 16, wherein the oligonucleotide material is added along with one or more of the aqueous solutions forming the particle, prior to the formation of the particles,

to form particles that have the polynucleotide material at least partially impregnated in the particle.

19 The method of claim 16, wherein the oligonucleotide material is small inhibitory RNA (siRNA).

20. The method of claim 16, wherein the particles are administered alone or in combination with a pharmaceutically acceptable carrier

21. The method of claim 16, wherein the particles are administered orally, intranasally, intratracheally, intrapulmonary, via aerosolization of particles containing biological payload, bucally, intraocularly, via an eye drop or an ointment, intratumorally, subcutaneously, or vaginally.

22. The method of claim 16, wherein the oligonucleotide e material is added in the form of a vector or naked DNA, along with one or more of the aqueous solutions forming the particle, whereby a calcium phosphate biodegradable matrix is formed around the vector or naked DNA, and the vector or naked DNA becomes at least partially embedded in or on the particle.

23. A method for inhibiting expression of a gene in a patient, comprising delivering a particle comprising calcium phosphate having a substantially spherical shape and a substantially smooth surface, the particle being at least partially coated, impregnated, or both, with a small inhibitory RNA sequence that is used to silence gene expression.

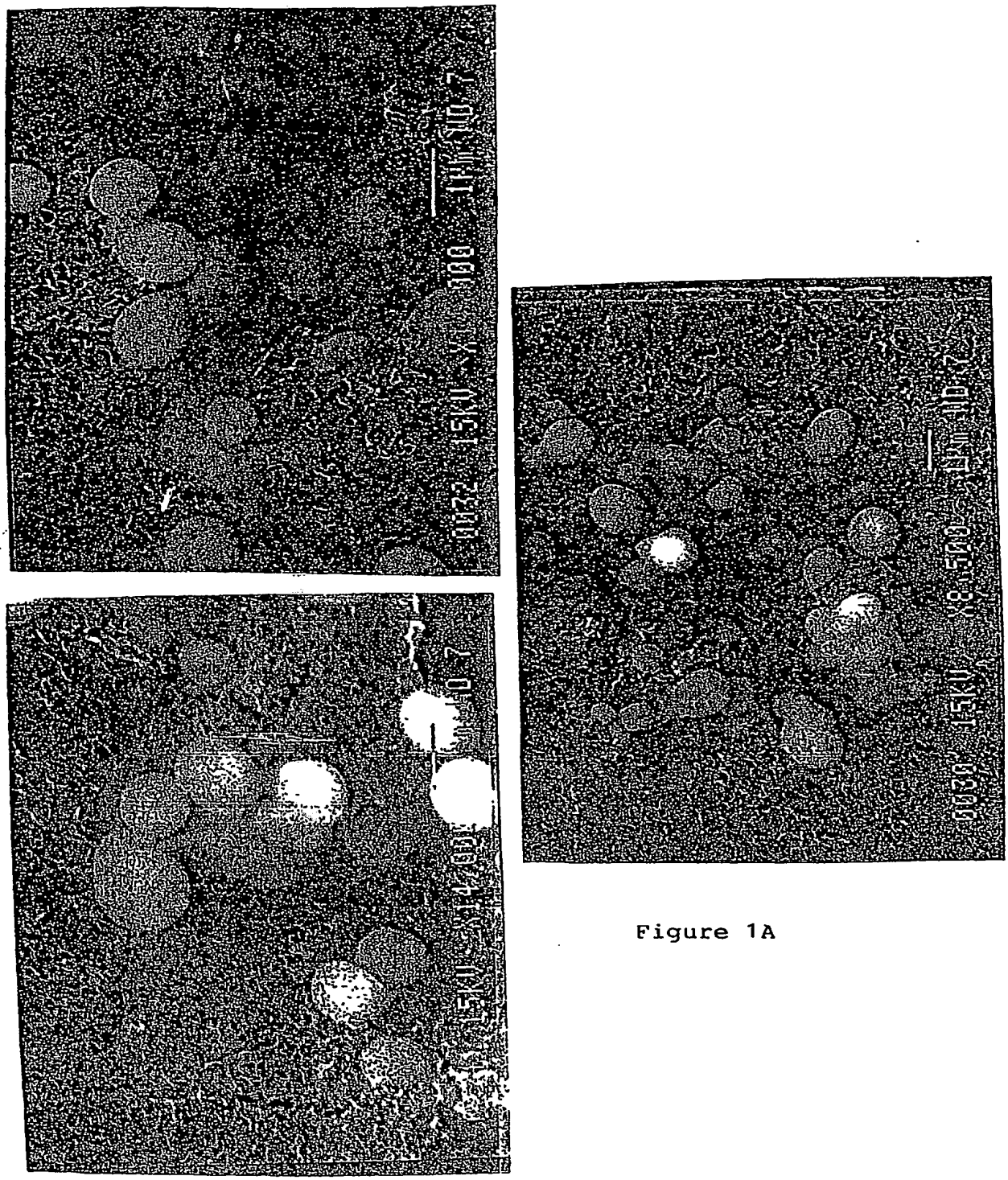


Figure 1A

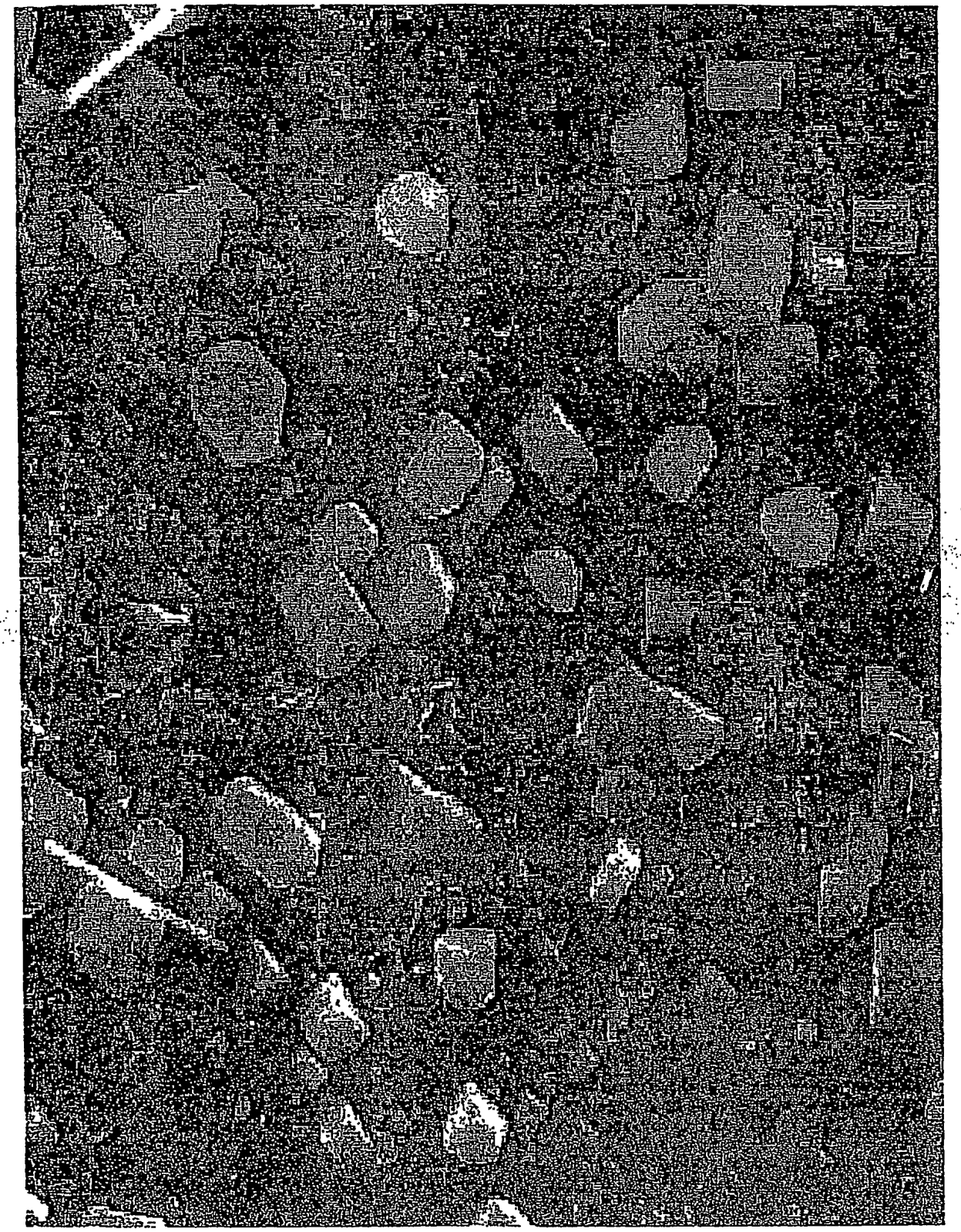
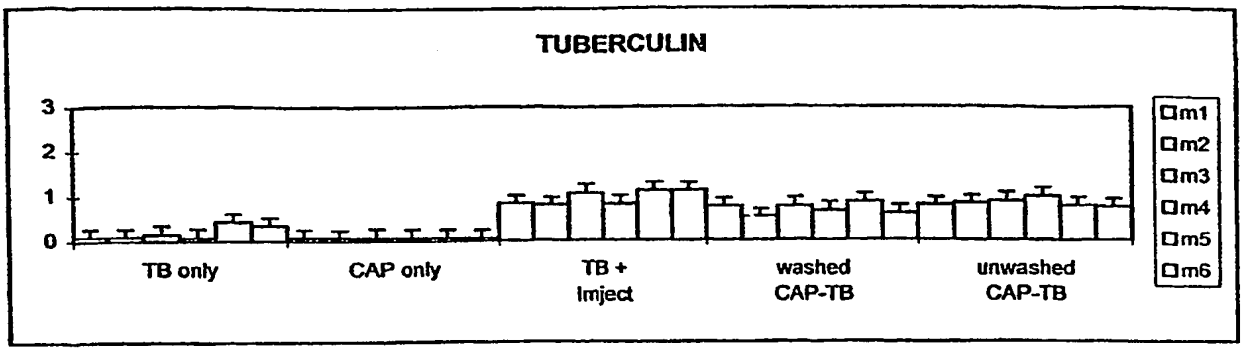
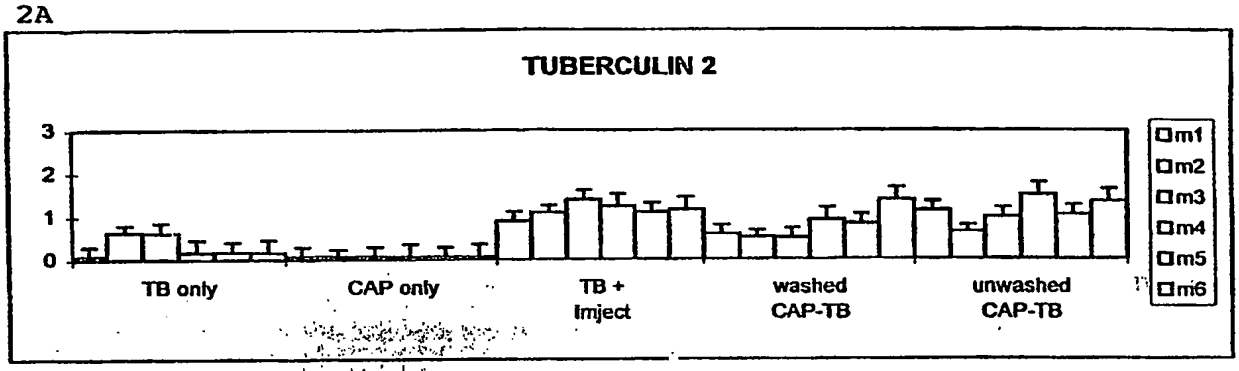


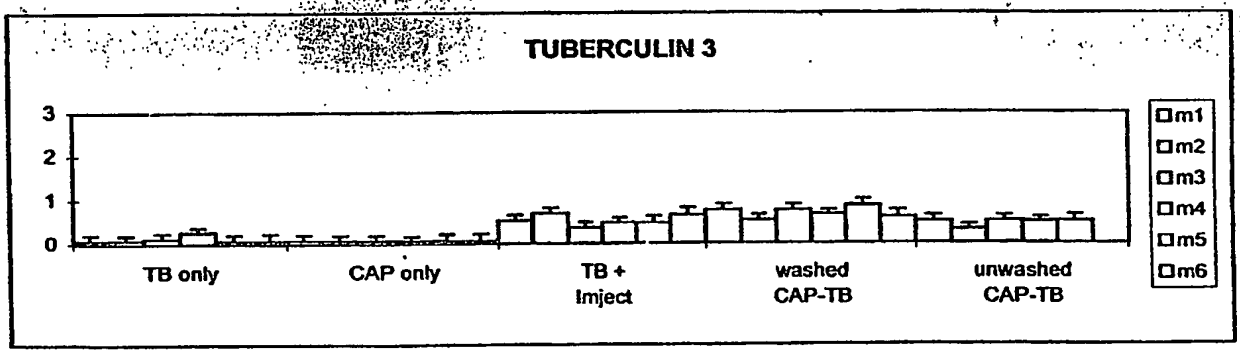
Figure 1B



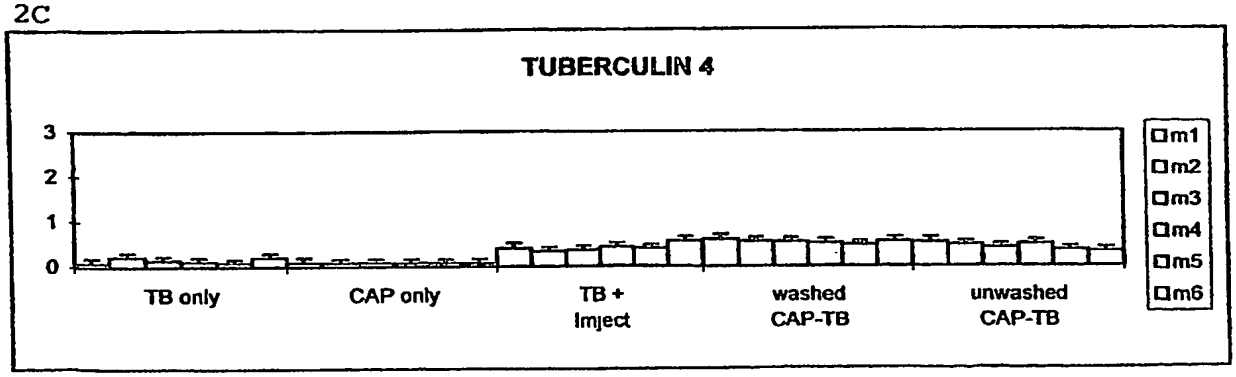
2A



2B

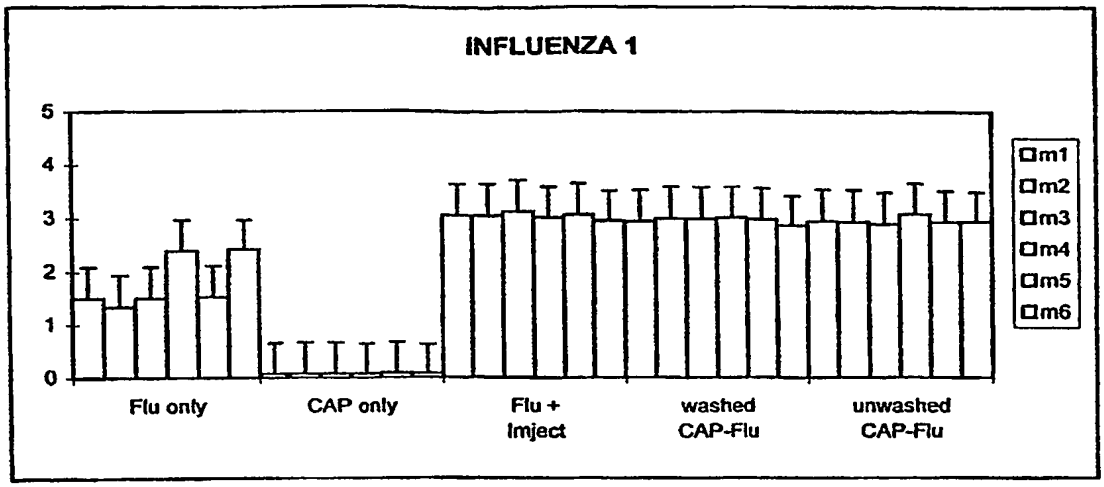


2C



2D

Figure 2



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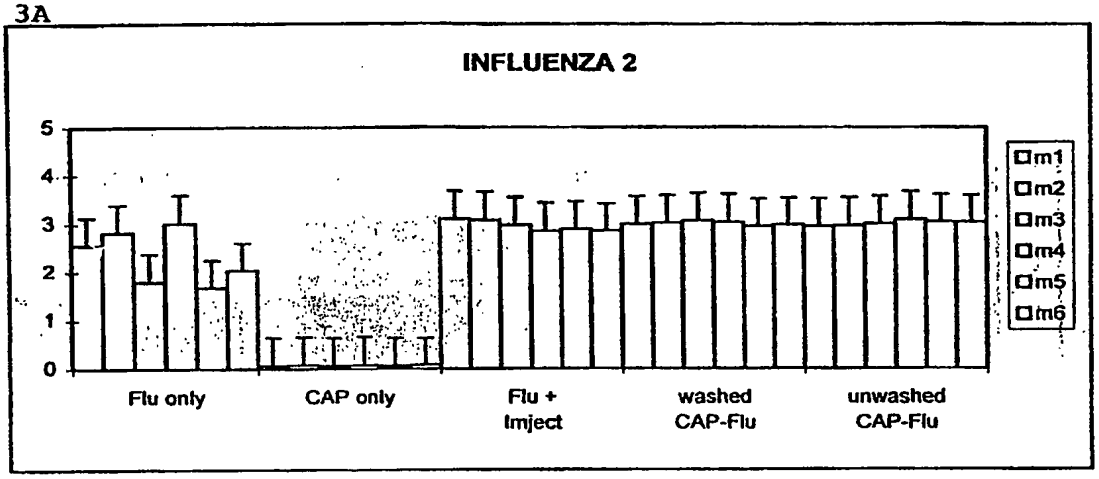
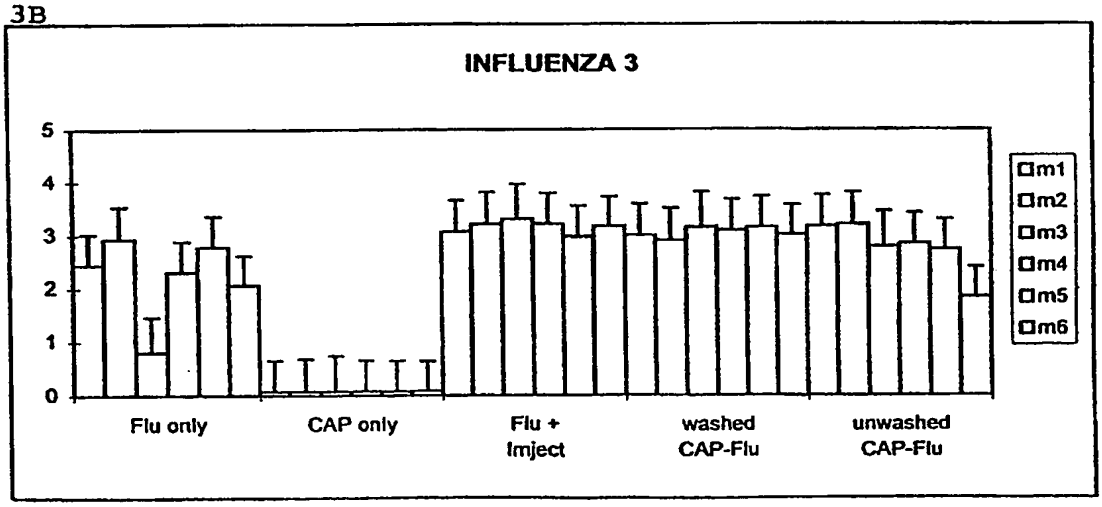


3B

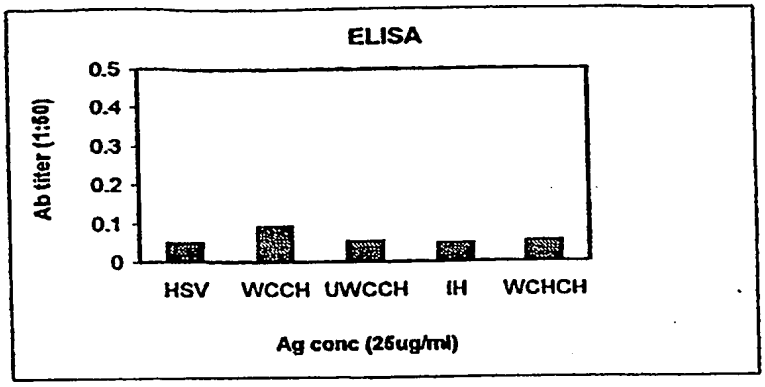


3C

Figure 3

7/13/98

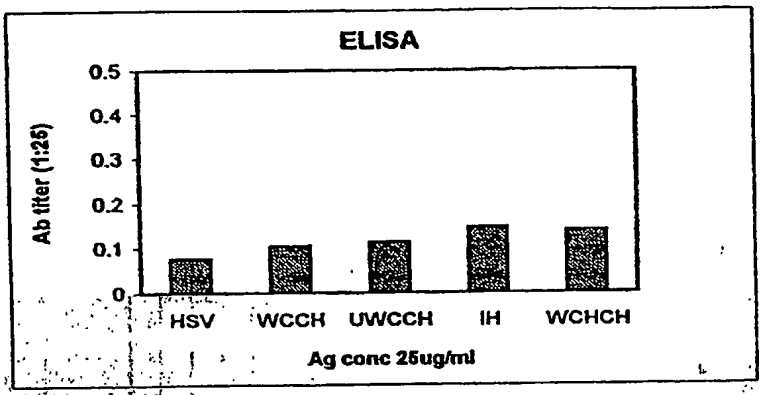
HSV	0.05
WCCH	0.092
UWCCH	0.052
IH	0.047
WCHCH	0.052



4A

7/29/98

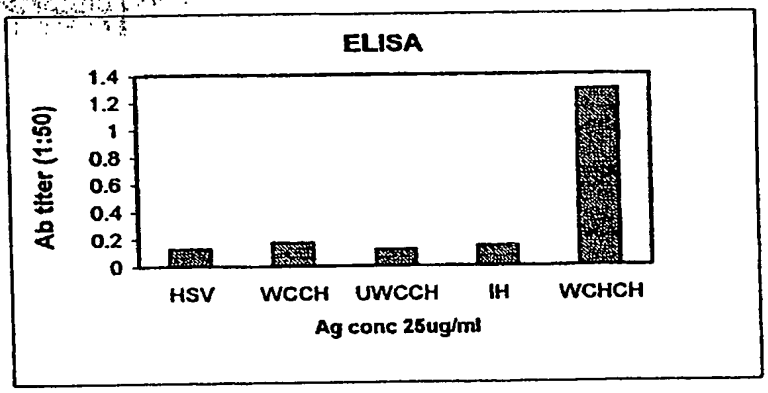
HSV	0.076
WCCH	0.105
UWCCH	0.114
IH	0.145
WCHCH	0.139



4B

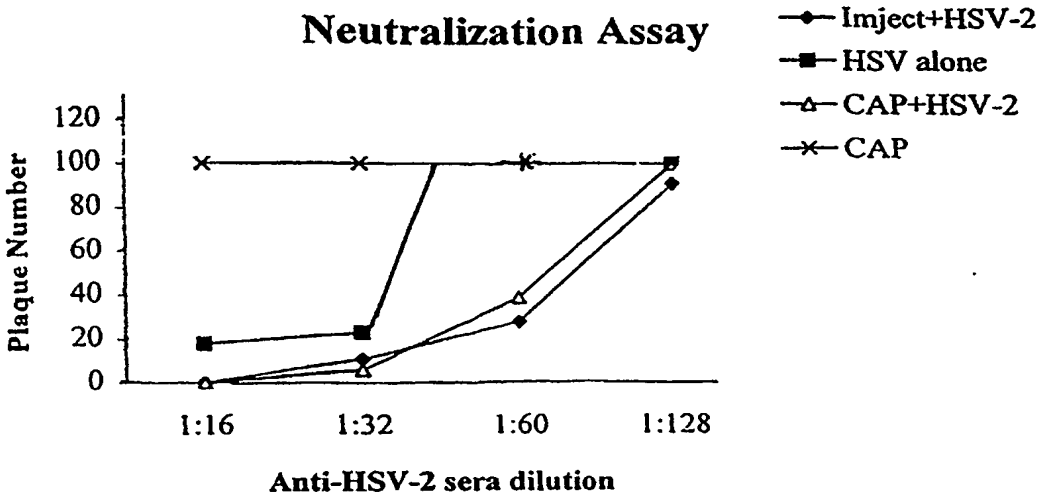
8/21/98

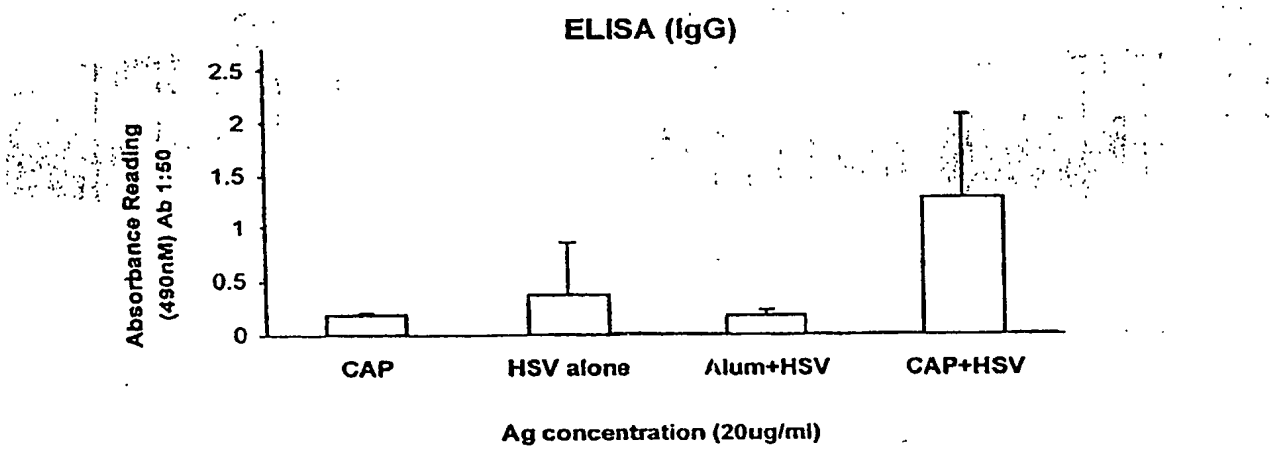
HSV	0.127
WCCH	0.169
UWCCH	0.121
IH	0.146
WCHCH	1.287



4C

Figure 4





Note

1. ^ First immunization was 6/22/1998. 1st boost was 7/16/98 2nd boost was 8/12/98

2. Each bar in graph represents the mean antibody titer of five mice in one group nine days after second boost

3 Antigen concentration for each group mouse is as follow

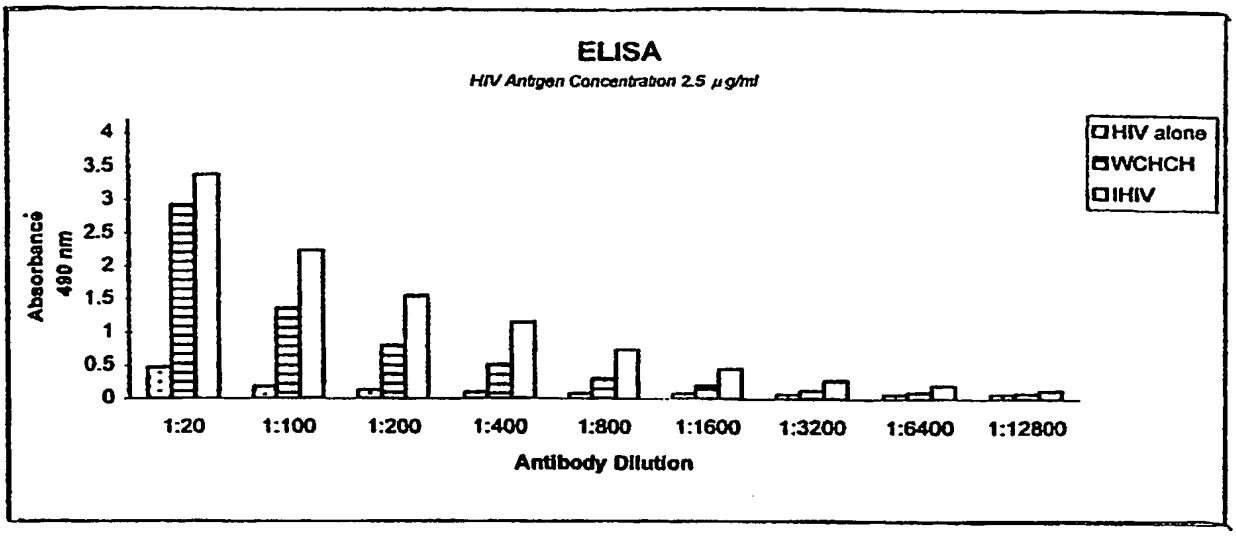
	6/22/1998^	7/16/1998^	8/12/1998^
HSV alone	44.5ug/mouse	52.5ug/mouse	129ug/mouse
IH	44.5ug/mouse	52.5ug/mouse	129ug/mouse
WCHCH	~8ug/mouse	~29.1ug/mouse	~59.2ug/mouse

4. CAP and Imject concentration for each group is as follow

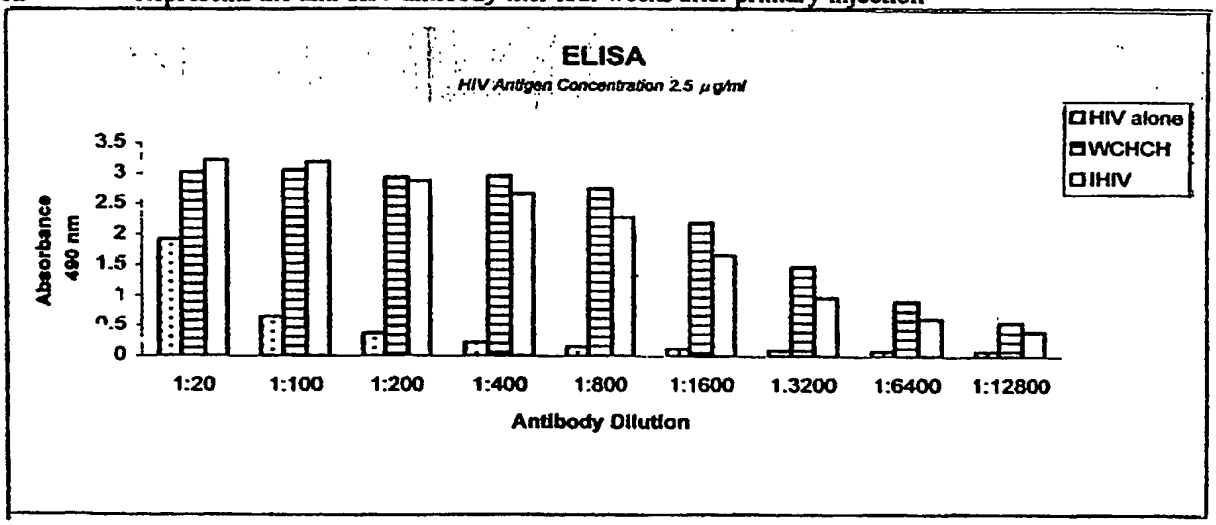
	6/22/1998^	7/16/1998^	8/12/1998^
IH	50mg/mouse	50mg/mouse	50mg/mouse
WCHCH	0.46mg/mouse	0.46mg/mouse	0.55mg/mouse

FIGURE 5

6A Represents anti-HIV antibody titer two weeks after primary injection



6B Represents the anti-HIV antibody titer four weeks after primary injection



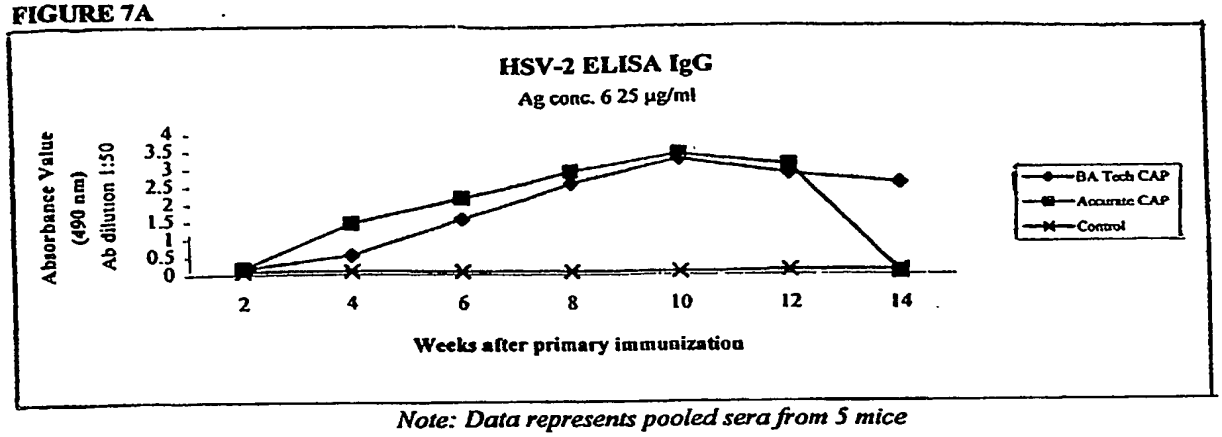
Note:

1. There are six mice in each group. The groups are: HIV alone (HIV), Cap+HIV (WCHCH), and Imject+HIV (IHIV).
2. The injection dosage for each group is as follows:

HIV: 6.9 ug HIV/ mouse
WCHCH: 9.5ug HIV/ 0.92mg CAP/ mouse;
IHIV: 6.9ug/ 50mg Imject/ mouse

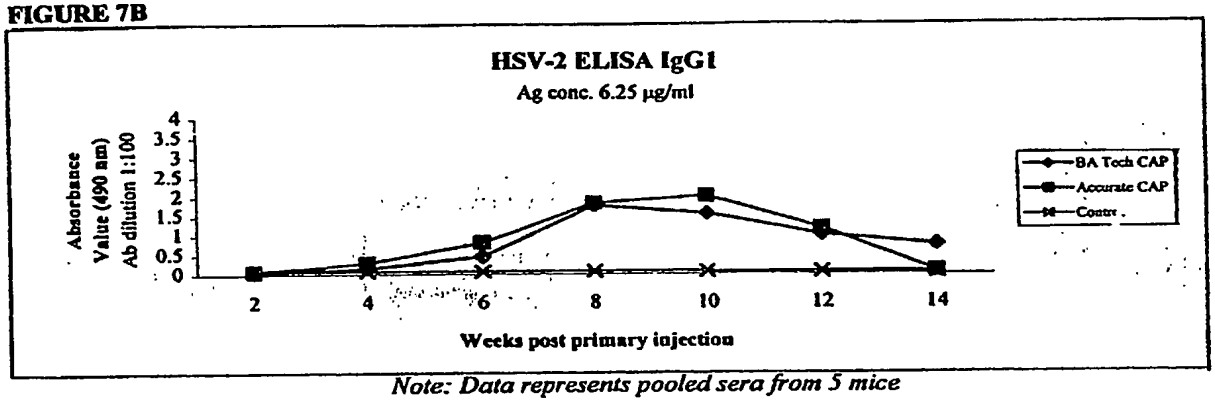
FIGURE 6

FIGURE 7A



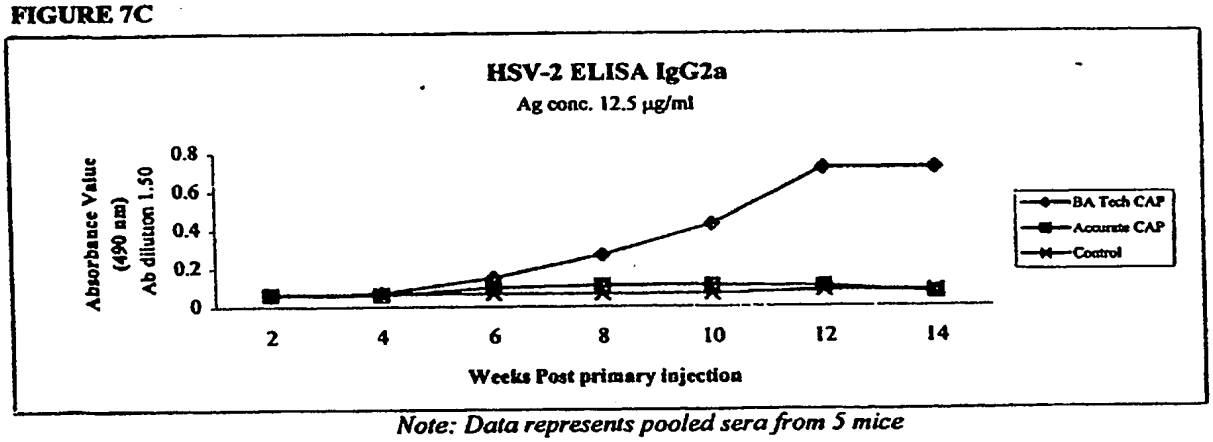
Note: Data represents pooled sera from 5 mice

FIGURE 7B



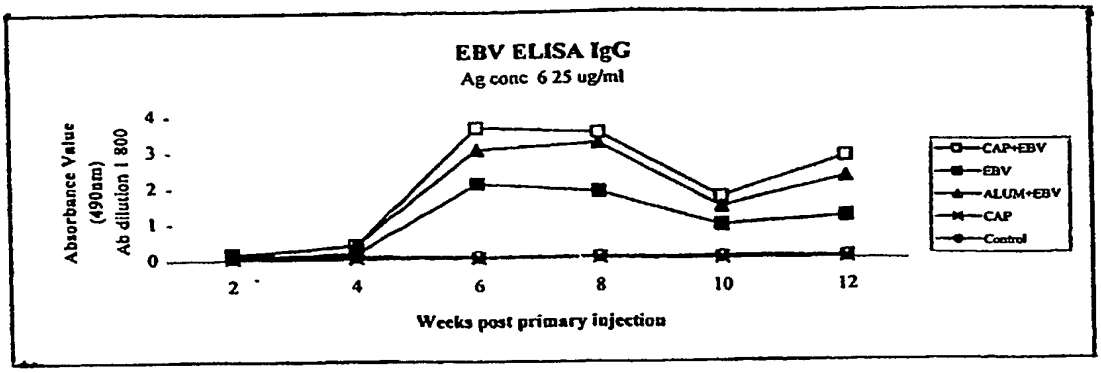
Note: Data represents pooled sera from 5 mice

FIGURE 7C

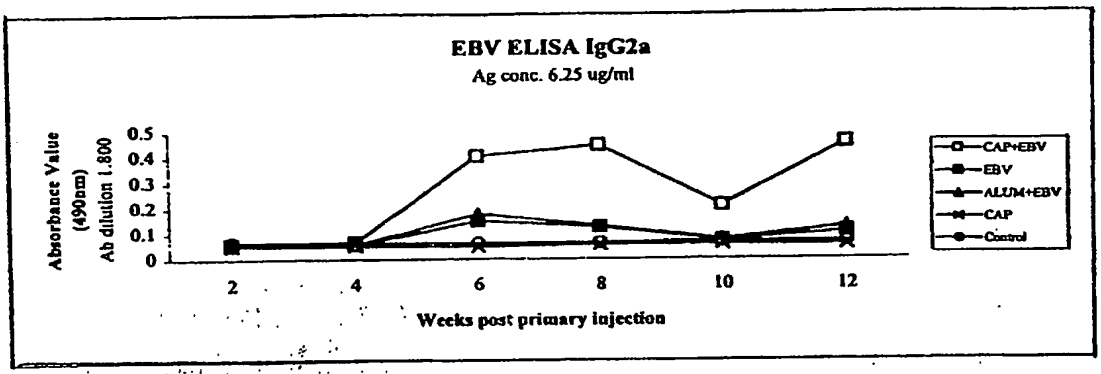


Note: Data represents pooled sera from 5 mice

8A



8B



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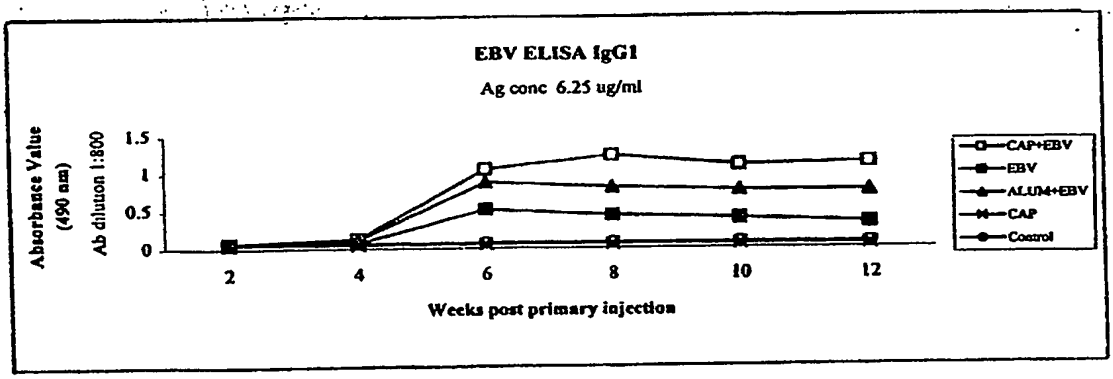


8D

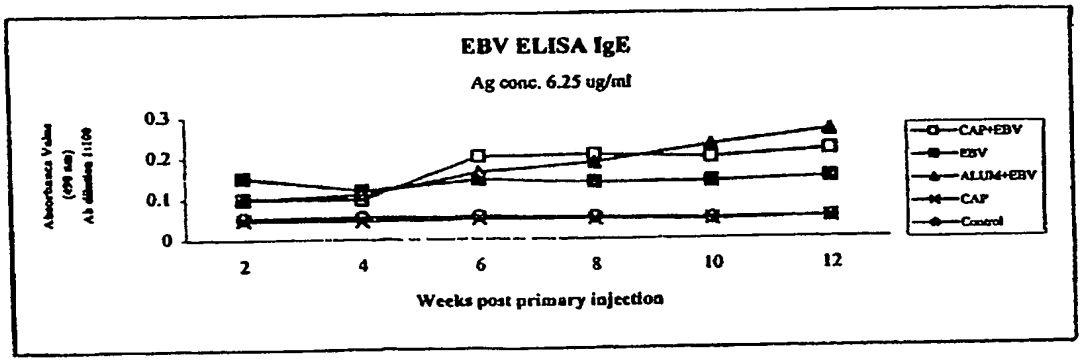
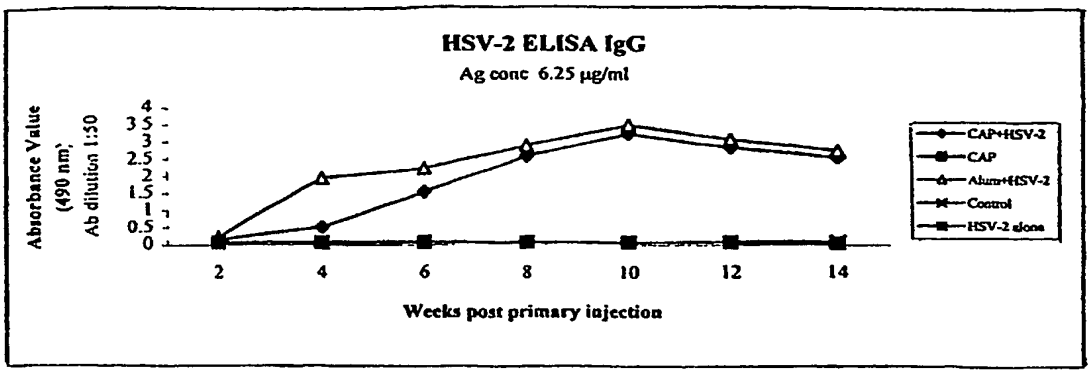


FIGURE 8

9A



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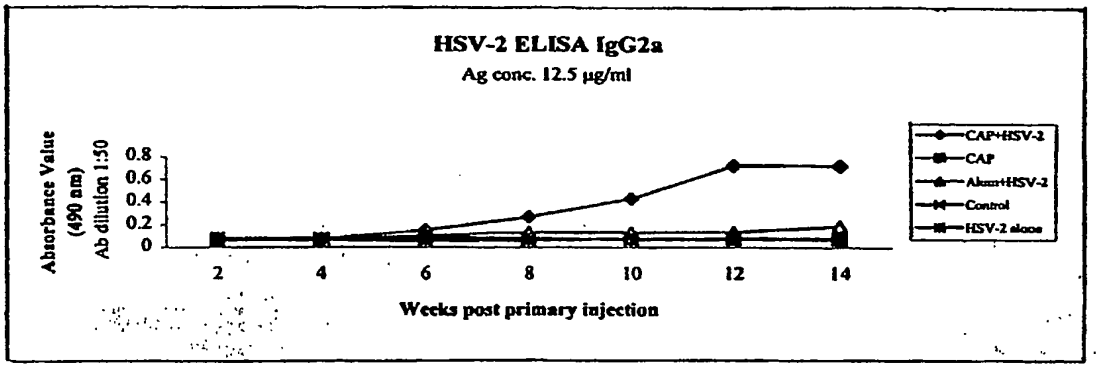

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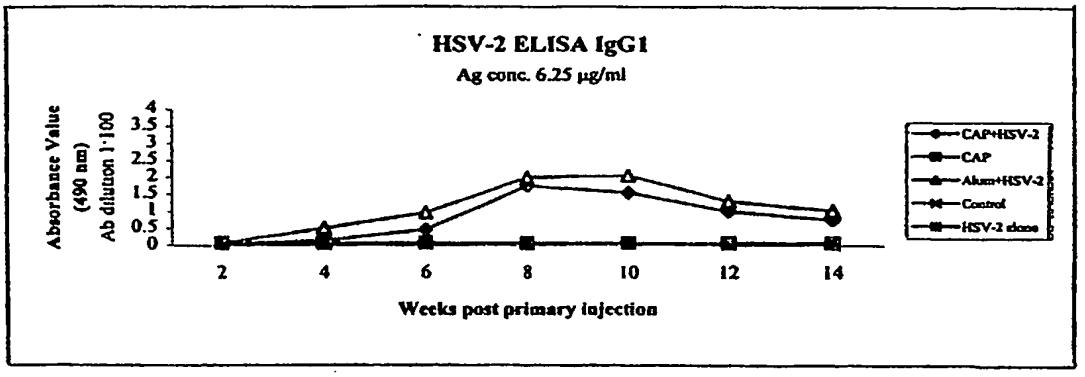

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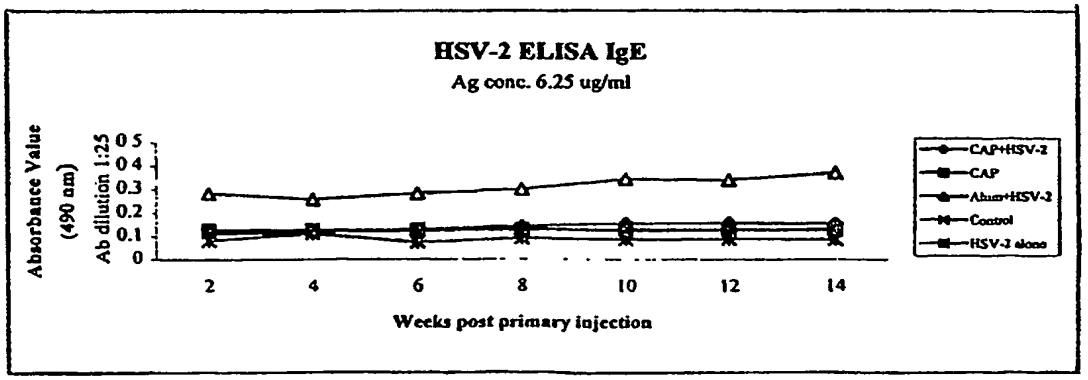
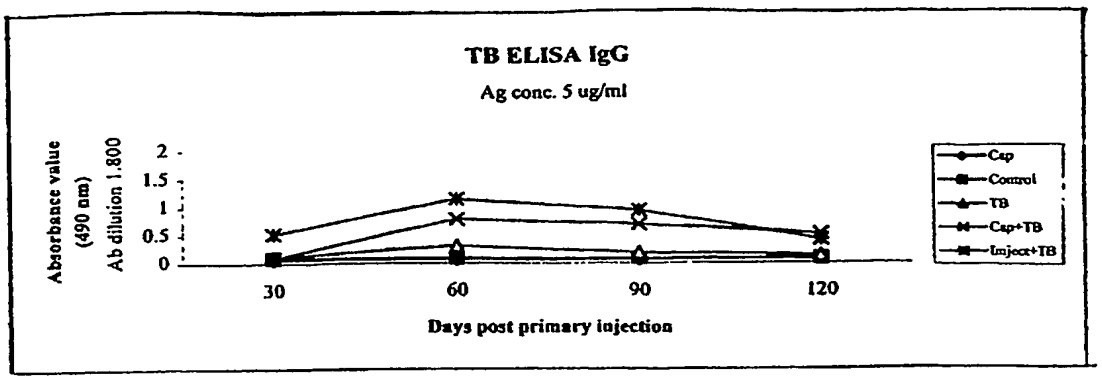


FIGURE 9

10A



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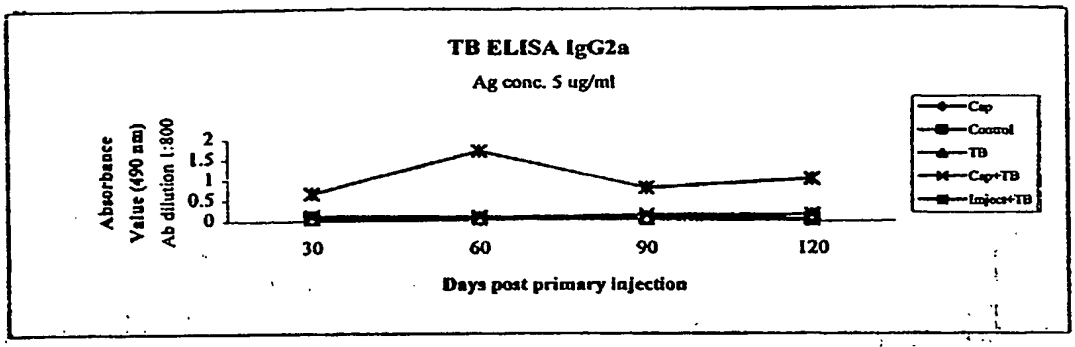

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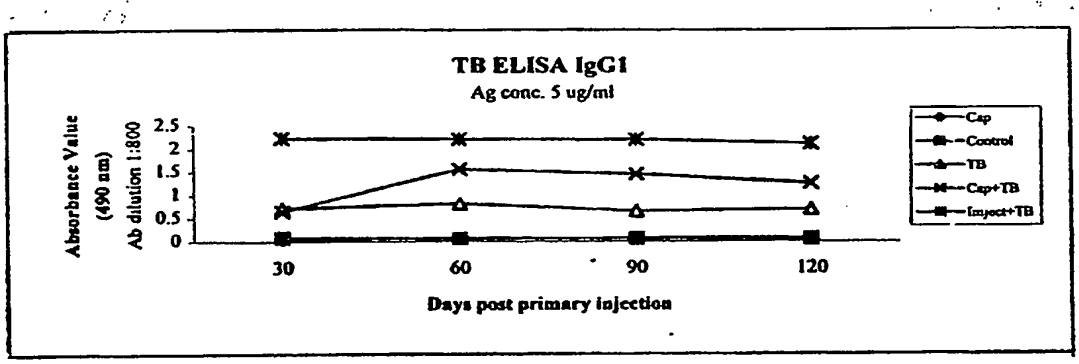

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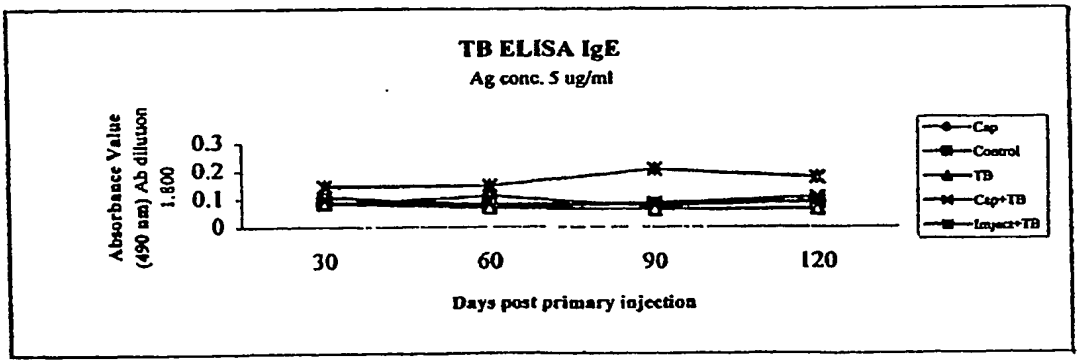
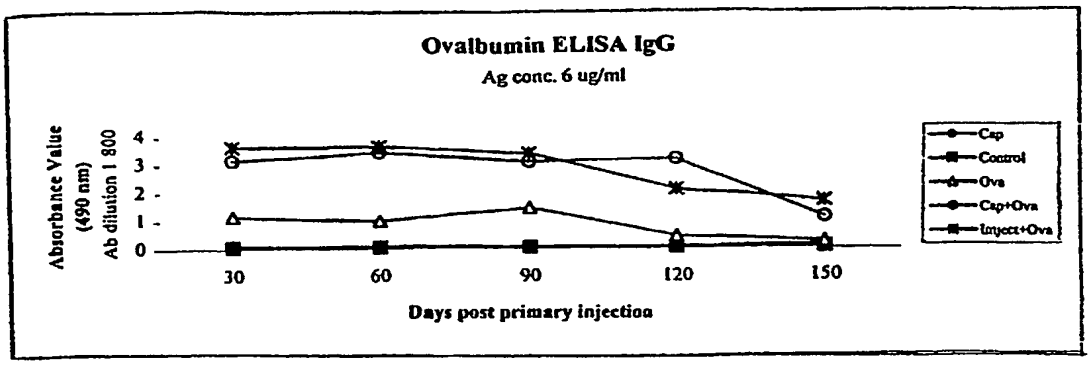


FIGURE 10

11A



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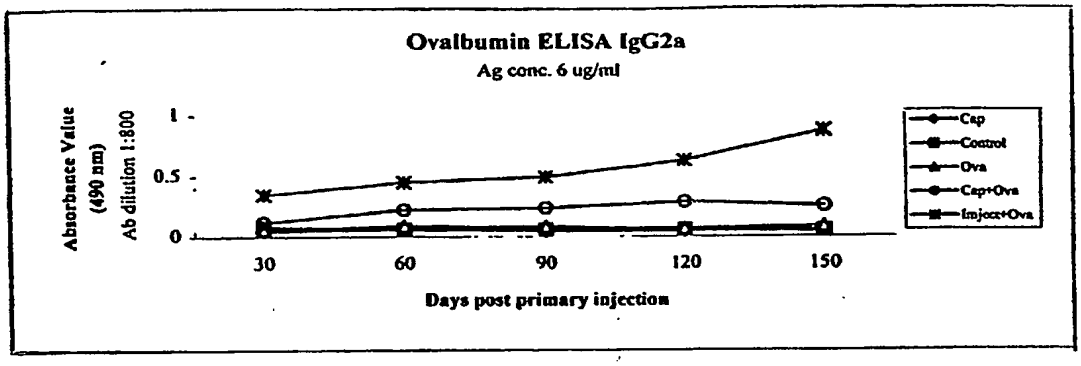
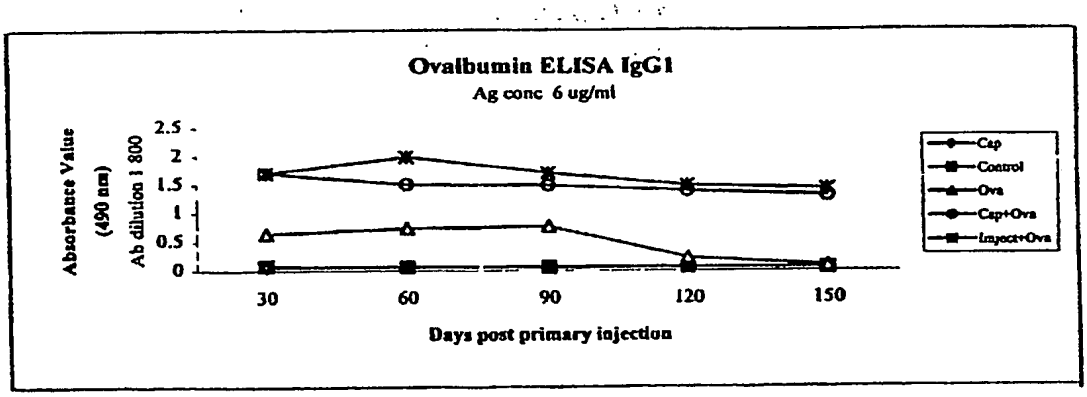


11C



11D

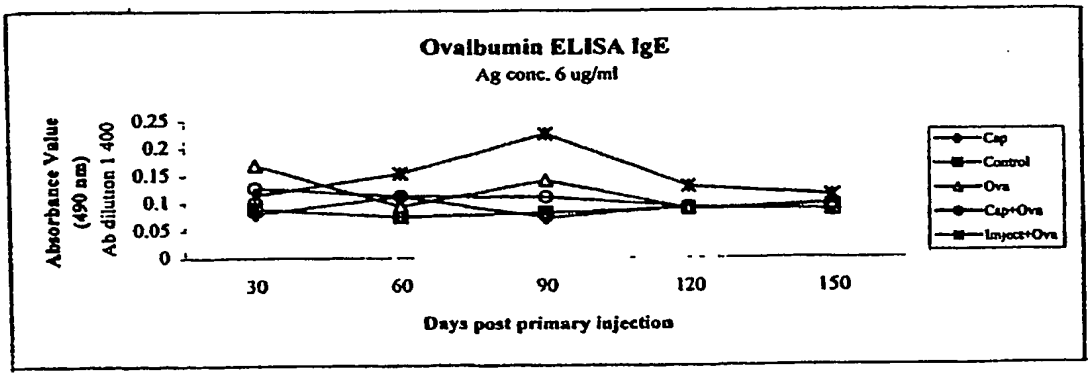


FIGURE 11

FIGURE 12

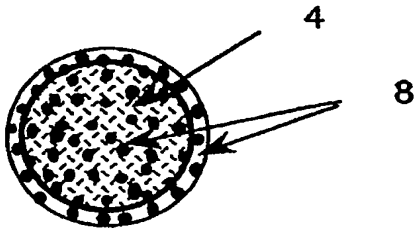


FIGURE 13A

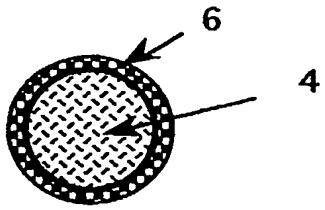


FIGURE 13B

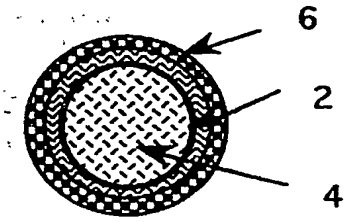


FIGURE 13C

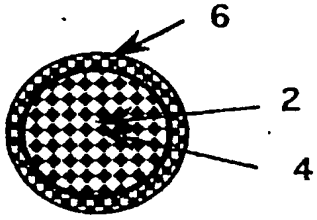
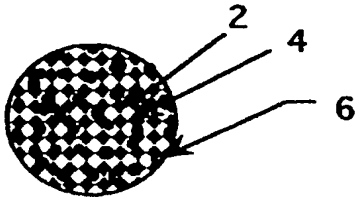


FIGURE 14



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/027519

A. CLASSIFICATION OF SUBJECT MATTER
A61K31/7105 A61K9/14 A61K9/51

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/46147 A (BIOSANTE PHARMACEUTICALS, INC; BELL, STEVE, J., D; WAGNER-BARTAK, CLAU) 10 August 2000 (2000-08-10) abstract page 7, lines 15-28 page 8, lines 24-31 page 14, lines 1-11,16-21 page 18, line 27 - page 21, line 3 claims	1-23
X	WO 03/051394 A (BIO SANTE PHARMACEUTICALS, INC) 26 June 2003 (2003-06-26) page 12, line 14 - page 13, line 10 page 20, lines 5-10 page 24, lines 10-31 claims	1-23

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 2005	29/12/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Villa Riva, A

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/027519

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 460 831 A (KOSSOVSKY ET AL) 24 October 1995 (1995-10-24) cited in the application column 3, lines 30-45 column 4, lines 15-60 claims	1-23
A	WO 2004/050065 A (BIOSANTE PHARMACEUTICALS, INC; CHU, TEH-CHING; POTTER, DAVID, E) 17 June 2004 (2004-06-17) the whole document	1-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/027519

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 23 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US2005/027519

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0046147 A	10-08-2000	AT 276199 T	15-10-2004
		AU 2753100 A	25-08-2000
		CA 2361421 A1	10-08-2000
		DE 60013773 D1	21-10-2004
		DE 60013773 T2	10-11-2005
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		EP 1418939 A2	19-05-2004
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		US 2003185892 A1	02-10-2003
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		US 5306508 A	26-04-1994
		US 5441739 A	15-08-1995
		US 5460830 A	24-10-1995
		US 5462751 A	31-10-1995
WO 2004050065 A	17-06-2004	AU 2003287726 A1	23-06-2004